Universidad Autónoma de Madrid
Facultad de Ciencias

Programa de Doctorado en Biociencias Moleculares

ANALYSIS OF THE DETERMINATION OF TESTIS DEVELOPMENT IN *DROSOPHILA MELANOGASTER*

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Madrid, 2017

Departamento de Biología Molecular

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Licenciada en Biología

Memoria presentada por Inés Olivera Crego para optar al grado de Doctor en Ciencias por la Universidad Autónoma de Madrid

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Madrid, 2017

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RESUMEN

El sistema reproductor de *Drosophila melanogaster* es un sistema complejo, derivado de la fusión de dos estructuras diferentes, las gónadas y el disco genital, y que se forma durante la metamorfosis. En esta etapa, los derivados del disco genital migran hacia delante desde la parte más posterior de la pupa hasta el nivel del quinto segmento abdominal, donde se encuentran las gónadas. Investigaciones previas, utilizando principalmente métodos quirúrgicos, sugirieron que la comunicación entre estas dos estructuras (disco genital y gónadas) era necesaria para el correcto desarrollo del sistema reproductivo, pero la necesidad o no de cada una de ellas para el desarrollo de la otra ha sido un aspecto controvertido. Usando mutaciones y otros métodos genéticos para modificar la actividad génica, hemos estudiado la participación de diferentes genes y tejidos en el desarrollo de los testículos. Hemos demostrado que el disco genital evagina de forma autónoma e independiente de las gónadas, ya sea en machos o hembras, pero que los testículos necesitan el contacto del disco genital para alargarse y espiralizarse. El músculo liso, procedente de este disco, rodea los testículos, que se alargan en coordinación con la migración del tejido muscular. El desarrollo normal de los testículos y, al menos en algunos casos, la especificación autónoma del músculo liso, depende de la expresión de *Abdominal-B*, *Sine Oculis 4* y *Drop*, de la activación de la vía de señalización de Notch y de que las células musculares sea de sexo masculino. Sin embargo, el desarrollo de los testículos también requieren información procedente del tejido adyacente, las Células Pigmentarias, que derivan del cuerpo graso y rodean al músculo. En estas Células Pigmentarias la vía Jun-quinasa está activa, y es necesaria para la especificación del músculo liso y la elongación de los testículos; como lo es también, así como ocurre en el músculo, la expresión de genes que determinan el sexo masculino. Localizados entre las Células Pigmentarias y los músculos, y entre las células musculares y las de la línea germinal, se encuentran componentes de la matriz extracelular, que también son necesarios para el correcto desarrollo de los testículos. Los defectos derivados del desarrollo de las Células Pigmentarias, la Matriz Extracelular o el músculo liso afectan a la fertilidad del individuo. Nuestro trabajo ha demostrado la importancia de diferentes genes y vías de señalización, actuando en músculos y Células Pigmentarias, para el desarrollo correcto del testículo y la fertilidad de los machos de Drosophila.

ABSTRACT

The reproductive system of *Drosophila melanogaster* is a complex system, derived from the fusion of two different structures, the gonads and the genital disc, and formed during metamorphosis. At this stage, derivatives of the genital disc migrate anteriorwards from the most posterior part of the pupa to the level of the fifth abdominal segment, where the gonads are located. Previous research, using mainly chirurgical methods, suggested that the communication between those two structures (genital disc and gonads) was necessary for the correct development of the reproductive system, but the need or not of each of them for the development of the other was controversial. Using mutations and other genetic methods to modify gene activity, we have studied the involvement of different genes and tissues in the development of the testicles. We have demonstrated that the genital disc evaginates autonomously and independently of the gonads, either in males or females, but that testes need the contact of the genital disc in order to elongate and loop. Smooth muscle, coming from this disc, surrounds the testes, which elongate in coordination with muscle migration. The normal testes development and, at least in some cases, the autonomous specification of smooth muscle, depends on the expression of *Abdominal-B*, *Sine Oculis 4,* and *Drop*, on the activation of the Notch signaling pathway, and on muscle cells having the male sex,. However, testes development also requires information coming from adjacent tissue, the Pigment Cells, which are derived form the fat body and surround the muscle,. In these Pigment Cells, the Jun-kinase pathway is active and necessary for muscle specification as smooth and testes elongation, and so is, as in muscle, an active male sex determination pathway. Localized between Pigment Cells and muscle, and between muscle and stem cells, there is Extra-Cellular Matrix components, which are also necessary for the correct development of the testes. The defects derived form alteration in Pigment Cells, Extra-Cellular Matrix or smooth muscle development have an impact on the fertility of the individual. Our work has shown the importance of different genes and signaling pathways, acting in muscles and adjacent Pigment Cells, for the correct testis development and the fertility of Drosophila males.

ABREVIATIONS

Abbreviation	
abd-A	abdominal A
Abd-B	Abdominal B
AEL	After egg laying
An	Analia
APF	After Puparium Formation
cad	caudal
Cad	cadherine
Cdc2	Cyclin dependent kinase, 1
Dr	Drop A.K.A. msh
ECM	Extra-Cellular Matrix
ed	Ejaculatory duct
ems	empty spiracles
eve	even skipped
FB	Fat-Body
FCMs	Fusion Competent Myoblasts
FCs	Founder Cells
GSCs	Germ Stem Cells
Ilk	Integrin Linked Kinase
JAK/STAT	Janus Kinase/ Signal Transducer and Activator of Transcription
JNK	c-Jun N-terminal kinases

Kr	Krüppel
lam	laminin
Lb	ladybird
msh	muscle homeotic gene
msSGPs	male-specific Somatic Gonadal Precursors
nau	Nautilus
O?	Ovary misssing
ov	oviduct
P	penis
PCs	Pigment Cells (testis)
pg	Paragonia
SGPs	Somatic Gonadal Precursors
Six-4	Sine oculis 4
sp	Spermatic pump
sr	Seminal receptacle
st	Spermatheca
T	Testicle
tin	tinman
vd	Vas deferens

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INTRODUCTION

I. 1 Developmental biology and Drosophila

At the beginning there was only darkness, nearly ten thousand millions years later there was a cell, the origin of all of us; hence, it can be said that every known cell derives from inexact copies of that very first one. Many processes and many millions of years were needed to create the vast diversity of life we observe today. It may seem irrational that life may manifest in so many different forms while sharing the fundamental basis; it is almost as "magical" as understanding how from a single cell, the zygote, all different specialized tissues that form a body may be originated from exactly the same genetic information. The key to understand evolution and development rests in niches and specialization. While living beings make use of some of the resources available in the environment and respond to it, cells do the same, they integrate the information from their environment and use the genetic information hosted in their nucleus differently from their neighbours. Each and every cell may respond differently to changes in their environment by finding their unique fate, which is the basis of specialization.

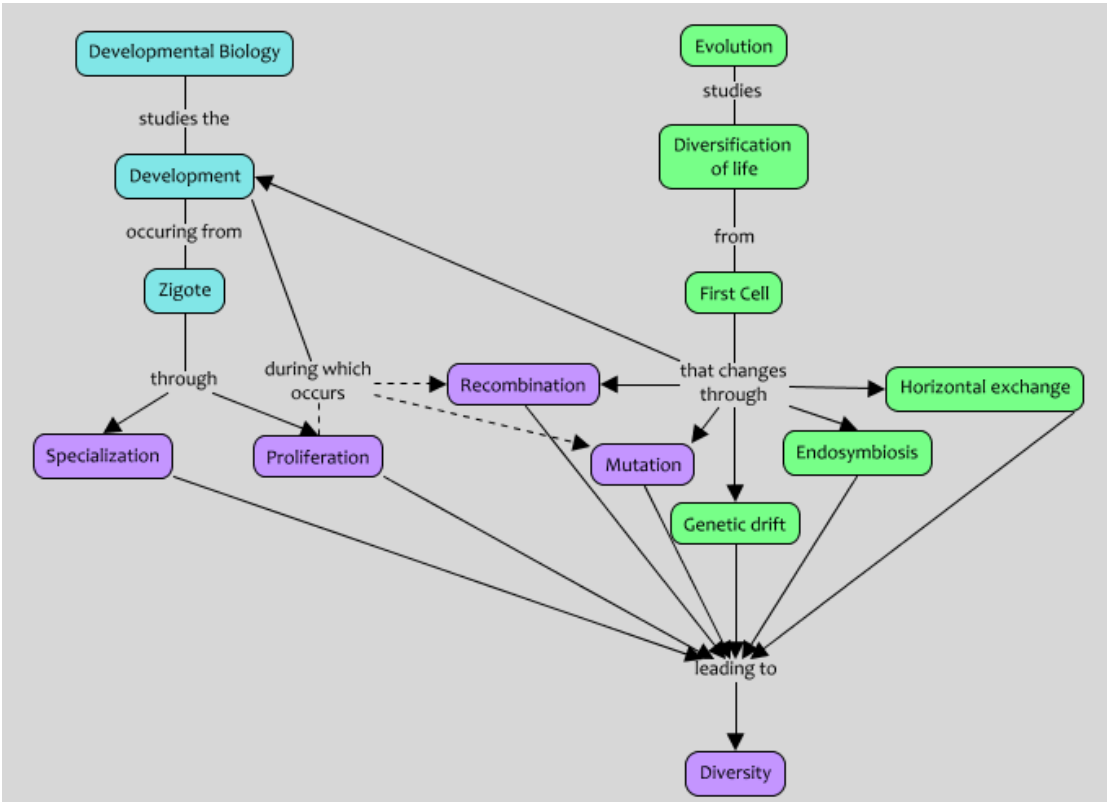


Fig. 1 Relation between Developmental biology and evolution leading to diversity.

Some mechanisms underlying evolution may be shared in the development of an individual (Fig. I. 1). During evolution, some processes such as endosymbiosis, mutation, recombination, genetic drift and horizontal exchange may operate together with proliferation and specialization, these two last processes being also common during the development of an individual; this is studied by Developmental Biology, the science that integrates concepts and techniques from Genetics, Molecular Biology or Compared Taxonomy to understand how a living being is formed.

During this construction of a living organism there are two basic procedures: cell proliferation and differentiation or specialization. There are as well some other mechanisms contributing to development: intercellular communication, migration, apoptosis and cell rearrangement. This simplicity in key mechanisms is also operating at the genetic level: a few sets of genes are crucial controllers during development and evolution, and changes in genes and mechanisms are exploited during these two processes.

The previously discussed genomic and mechanistic conservation has helped scientists to select different animals as model organisms, which have been chosen by reason of their easy handling, phylogenetic proximity, and/or in-depth knowledge. Results obtained in these animals have been extrapolated to the rest of the animals, including humans. In the present work we have used as a model organism the fruitfly, *Drosophila melanogaster*, which has been intensively and extensively used by geneticists and biologists in general. *Drosophila* is easy to breed and handle, it has a short life cycle that allows observing many generations in a reduced period of time, a simple genome stored in four chromosomes and a wide range of genetic tools at our disposal. Also, the whole *Drosophila* genome has been sequenced, found to have a small number of repetitive elements, and many of its genes characterized, cloned and "tooled" allowing unique manipulations among the multicellular organisms (Spradling 1997; Jennings 2011; del Valle Rodríguez, et al. 2012; Hales et al. 2015)

I. 2 Anatomy and life cycle

Drosophila is a holometabolous insect, therefore it suffers a complete metamorphosis, which means that its adult form is different from the embryonic or larval configuration. At its growth optimal temperature, 25ºC, *Drosophila* needs ten days to complete its life cycle, which can be divided in four different phases. The first 24 hours cover the embryonic

development; this is followed by three larval stages along the next 3-4 days, and lastly, the pupal stage occurs, and this takes 5 days. After those nine to ten days, the adults will emerge from the pupae, they will be sexually mature within the next 10-12 hours and approximately two days after females will lay fertilized eggs to restart the cycle (Fig. I. 2 A).

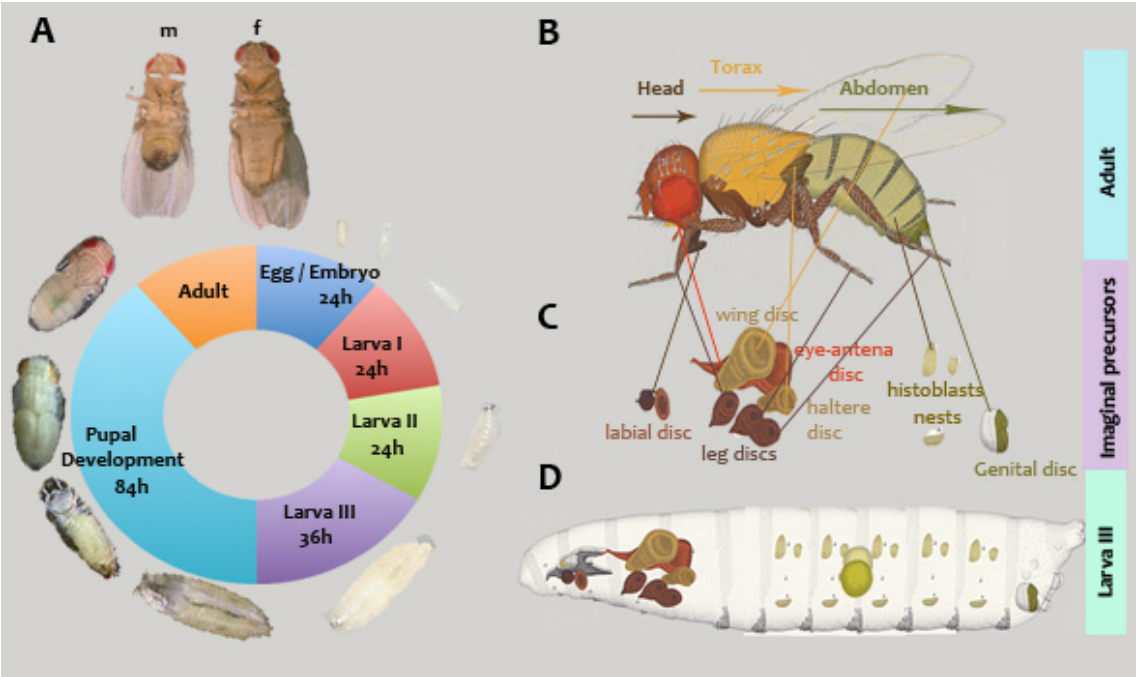


Fig. 2 Drosophila bodyplan changes through life cylcle.
A. Drosophila melanogaster life cycle. B-D. Body plan of Drosophila melanogaster at larval (D) and adult stages (B). Imaginal discs have been highlighted (C) and are connected to the structure they will give rise to. Modified from (Hartenstein, 1993).

The most dramatic changes occur during pupal stages, when imaginal tissues replace larval ones. Those imaginal tissues come from imaginal precursors that are already singled out at the embryo. There are two types of imaginal tissues making the epidermis, the histoblasts nests that start to divide only after the ecdysone pulse, which triggers metamorphosis, and the imaginal discs, which grow mainly during the larval period (Fig. I. 2. C).

As an arthropod, *Drosophila* presents a segmented body plan and its segments are defined at the embryonic stages. Segmentation is maintained throughout development and the adult body of *Drosophila* is organised in a cephalic tagma, with six segments, a thoracic tagma, composed of three segments, and a abdominal tagma that includes eleven segments, though only ten are distinguishable, as the last one is fused to the preceding one (Ferris 1950; Matsuda 1976; Jürgens 1987; Jürgens and Hartenstein, 1993) Only the last tagma, the abdomen, lacks appendages, and the adults present an abdomen with only six

segments in males and seven in females, the fifth and sixth male segments being heavily pigmented. (Fig. I. 2. B). Though conspicuous, this is not the unique sexual dimorphic feature: sex combs are only found in the male first pair of legs, and the key element of the sexual dimorphism, the reproductive system, also differs from one sex to the other.

From the embryonic stage every segment is divided into anterior and posterior compartments, independent units of cell lineage (García-Bellido et al., 1973). The segments are determined at the embryo by a genetic cascade including: first, maternal effect genes, later gap and pair-rule genes, and finally segment polarity and Hox-genes (Gilbert, 2000). The latter specify different structures along the anteroposterior axis in bilaterians. They code for proteins activating or repressing other genes during development and when mutated display altered and/or misplaced body parts (Mann et al., 2009; Rezsohazy et al., 2015). In Drosophila, they are organized in two genetic complexes: Antennapedia and Bithorax. The latter determines the posterior part of the body and it is composed of three different Hox genes: *Ultrabithorax* (*Ubx*), *abdominal-A* (*abd-A*) and *Abdominal-B* (*Abd-B*) (Sanchez-Herrero et al., 1985). *abd-A* and *Abd-B* determine the segments where the reproductive system form.

This reproductive system is different from male to females, therefore, it would be interesting to understand how the sex is determined in *Drosophila melangaster*.

I. 3 The reproductive system of *Drosophila melanogaster*.

The reproductive system of *Drosophila melanogaster* is a complex structure, derived from the fusion of two organs: the gonads and the imaginal genital disc. Gonads are located in the adult abdominal segments 4 to 5 (A4-5), whereas genital disc lies in abdominal segment 8 (A8). The contact between these two components occurs during pupal stages (Dobzhansky, 1930; Dobzhansky and Tan, 1937; Epper, 1983a, b; Szabad and Nöthiger, 1992).

I. 3.1Sex determination in the reproductive system

The theories about *Drosophila* sex determination have changed from the initial one in which the X chromosomes/autosomes ratio (X: A) would trigger the sex determination cascade in the whole body (Bridges, 1921; Bridges, 1925), to a more recent and restricted definition. First, instead of being active in each cell, the sex cascade would be only activated in the sex-specific tissues such as the reproductive system, sex-combs and fat body (Hemple and Olivier, 2007; Robinett et al., 2010).

In females, an X:A rate of 1 triggers the transcription of *Sex lethal* (*Sxl*), which in turn determines the specific splicing of the *transformer* (*tra*) mRNA to form the Tra protein. This, together with the Tra-2 protein (also present in males) specifies the splicing of the *double-sex (dsx)* and *fruiteless (fru)* genes to the female forms (DsxF and Fru). In males, a X:A ratio of ½ fails to activate *Sxl* or splice *tra* into a protein-coding mRNA, and the *dsx* gene is spliced to the default form, making the DsxM protein. The Dsx and Fru proteins would, in turn, mediate the expression of downstream effectors controlling sexual behavior and morphology, such as pigmentation and reproductive system development (Christiansen et al., 2002).

In the reproductive system, the sex determination pathway determines, in the genital discs, a differential growth of primordia in males and females (Epper & Bryant, 1983; Sánchez & Guerrero, 2001), a male-specific cell migration and mesodermal to epithelial transformation (Ahmad and Baker, 2002), and the modulation of the organizers *wingless* (*wg*) and *decapentaplegic* (*dpp*) (Keisman and Baker, 2001). In the gonads, the sex-determination route allows the maintenance of somatic cells: for example, *Sxl* mutants show no gonads due to a problem of sexual identity (Siera and Cline, 2008). It has been also pointed out that this phenomenon is a non-autonomous effect: germ-line cells and gonads are sensitive to their environment and therefore respond to it accordingly (DeFalco et al., 2008; Murray et al., , 2010; Siera & Cline, 2008)

The differences in the reproductive sistem between males and females will be explained in what follows.

I. 3.2 The gonads: where gametes are produced

Gonads are mesodermal structures localized in the A4-5 segments that require an orchestrated genetic and cellular input to form. They contain the germ cells, which derive from the pole cells, a small group of cells formed at the back of the precellular blastoderm that migrate over the mesoderm during germ band extension. A small population of pole cells, the Germ Stem Cells (GSCs), will be ensheathed by Somatic Gonadal Precursors (SGPs), which derive from mesodermal primordia of parasegments 10-12 (segments A4-A7), form the gonadal sheath and are attached to the Fat-Body (FB). In males, there is also a male-specific type of Somatic Gonadal Precursors (msSGPs), derived from PS13 (A8) that first coalesce with Germ Stem Cells (GSCs) and later both do it with SGPs. After coalescence, in males, some of the FB cells will differentiate into a particular type of cells, Cells (PCs), which will ensheath the gonad.

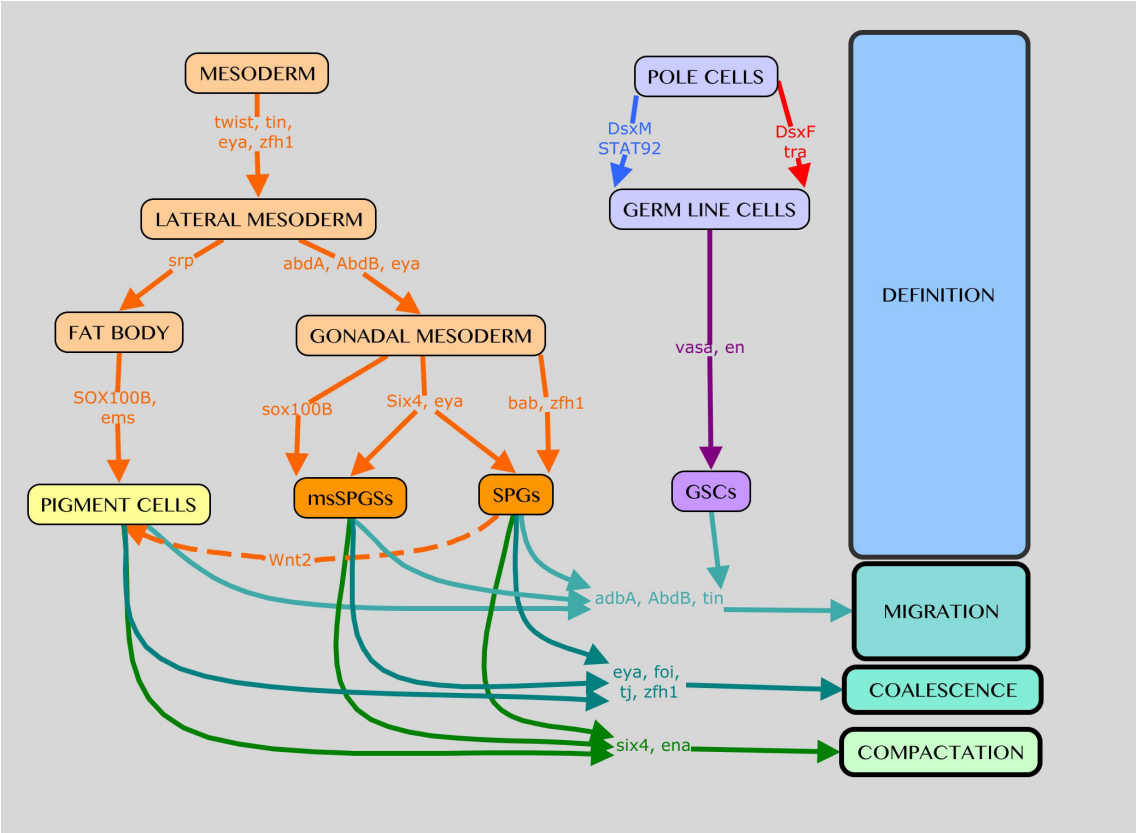


Fig. 3 Gonad formation.

Two populations are needed to form the gonads: somatic gonadal mesoderm, and pole cells, which will give rise to the germ line cells. In the case of males, another two populations are present: male specific Somatic Gonadal Precursors (msSGPs) and Pigment cells (PCs). On the right, the main steps are summarized inside the rectangles. On the left, in rectangles, cells and tissues involved. The connecting words are the genes required in each step.

The genetics behind gonad formation includes first the definition of each population before coalescence (Fig. I. 3). SGPs are specified at stage S15 by *eyes absent* (*eya*), *Six4*, (both genes required also in the formation of the eye), and the Hox genes *abd-A,* determining anterior, and *Abd-B*, specifying posterior, SGPs (DeFalco et al. 2003; Clark et al. 2007; Camara et al., 2008) It is important to note that SGPs are sexually determined, as they control, only in males, the development of msSGPs and the hub, the niche of the GSCs. msSGPs are specified at stage S13, and just like SPGs their fate depends on *eya Six4* and *Abd-B* but not *abd-A*, and express the transcription factor Sox100B as well (DeFalco et al. 2003; Clark et al. 2007; Camara et al., 2008). These msSGPS are initially formed in both sexes, but soon disappear in females by apoptosis; in male adults, they give rise to the terminal filament of the testis, which is in direct contact with the vas deferens, a structure derived from the genital disc (Nanda et al. 2009).

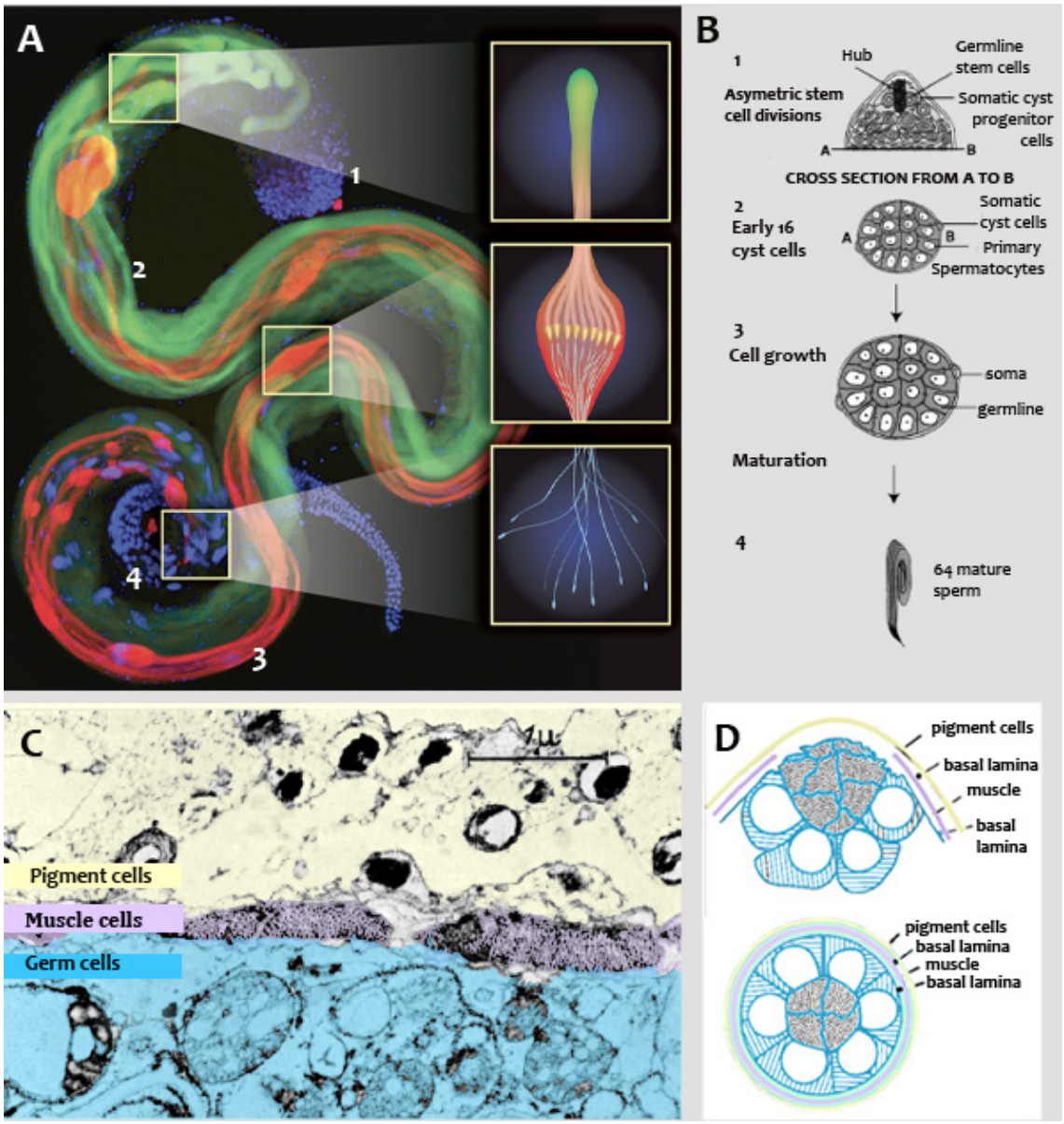


Fig. 4 Testis structure.

A. Longitudinal section of and adult testis of Drosophila melanogaster depicting four stages of the spermatogenesis occurring along the antero-posterior axis. Four portions can be identified: 1. The Hub, where the GSCs are found and marked here by fasciclin III staining (red dot), 2. Spermatogonia region. 3. Spermatid domain. 4. Spermatozoids (from Arama lab, http://www.drosophila-images.org/2011.shtml). B. Schemes of transversal sections of an adult testis. The number and stages correspond to those in A (from Williamson and Lehmann, 1996). C. Dorso-ventral section of an adult testis of Drosophila. Electronic microscope image depicting PCs in yellow, muscle cells in pink and germ cells in blue (modified from Bairati, 1967) D. Schemes of longitudinal and transversal section of the hub region of the testis, depicting the two different tissues separated by a layer of Extra Cellular Matrix (ECM). PCs in yellow, muscles in pink, ECM in green and germ cells in blue (modified from Hardy et al., 1979).

In the adult stage, testes are 1.9 mm long and 90 to 110 µm wide compact, coiled structures where the maturation of the sperm takes place along their whole length. From the hub region (Fig. I. 4 A.1 red dot, and B.1) to the vas deferens region, two areas may be

recognised: a coiled part where spermatocytes may be observed (Fig. I. 4 A.2 and B.2) and an elongated portion containing spermatogonies, cysts and spermatids (Fig. I. 4 A.3 and B.3) (Miller & Pitnick, 2002).

The germinal tissue is obviously well protected, first, at larval stages, by a PC layer and later, in the adult, by two layers of tissue, PCs and muscle (Fig. I. 4 C) and two basal laminas (Fig. I. 4 D). The most exterior tissue is a sheet of PCs, underneath which there is a basal lamina, below which are the muscle tissue and their own basal lamina, which, in turn, surrounds the different stem-cell populations (somatic and germinal), except in the hub. This latter fact means that GSCs are in direct contact with pigment cells (Hardy et al., 1979), therefore allowing a direct communication between them (Fig. I. 4 C and D).

I. 3.3The genital disc: the link between the inside and the outside.

The genital disc is an imaginal structure derived from the invagination of genital disc precursors from the last three abdominal segments, A8, A9 and A10, followed by their fusion and later localization at the A8 larval segment (Nöthiger et al., 1977; Schüpbach et al., 1978). The early development of the genital disc occurs similarly in both sexes, however, at the third larval stage, male and female discs present clear morphological and developmental differences. While the A10 segment gives rise to the analia and hindgut in both males and females, the A8 and A9 grow differently depending on the sex. Females show an A8 primordium bigger than the A9, whereas in males it is the A9 the bigger segment. In both cases the larger primordium will give rise to the external and most of the internal genitalia. The female A9 will form part of the uterus and the parovaria, which are accessory glands, and the A8 in males will develop a small non-sclerotized external A8 segment (Keisman et al., 2001; Nöthiger et al., 1977). (Fig. I. 5)

In the males, the genital disc differentiate: two structures to produce seminal fluid, the paragonia, two vas deferens (also known as vasa defferentia), and whose region most proximal to the testis, connecting them with the ejaculatory duct and the outside is the seminal vesicle), and the spermatic pump, that propels semen through the penis (Fig. 5) (Miller, 1941). In females, the ovaries are attached to the oviducts that are fused in one main conduct connecting to the uterus, that, in turn, connects to the exterior by the vagina. They also display different structures to preserve sperm, seminal receptacle and spermathecae, and the accessory glands, parovaria, of unknown function (Fig. 5) (Epper, 1983a; King, et al., 1968).

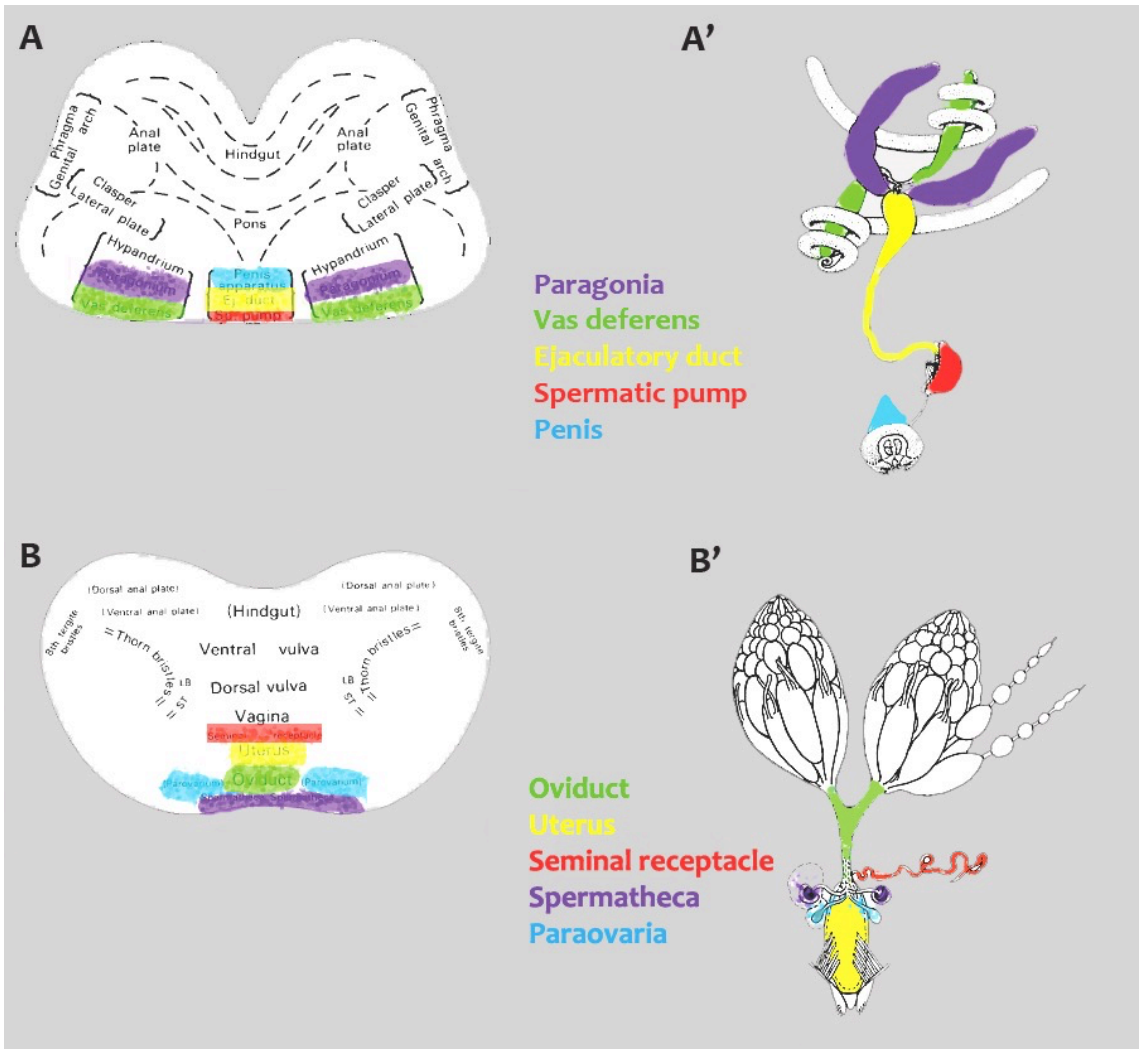


Fig. 5 Genital disc and its derivatives.

Fate map of the male (A) and female (B) genital discs, using the same colour code as in the adult genitalia (A', B'). Colour code as stated: Male: paragonia in purple, vas deferens in green, ejaculatory duct in yellow, spermatic pump in red and penis in blue. Female: oviducts in green, uterus in yellow, seminal receptacle in red, spermathecae in purple and parovaria in blue. Modified from (Epper, 1983b).

All theses structures can be distinguished since 12h After Puparium Formation (APF) but they develop fully only after adult eclosion; in fact, some structures, like oviducts, only finish their development after the first copula (Heifetz and Wolfner, 2004; Kapelnikov et al., 2008a). There are also some other changes affecting fertility that are produced after emerging from the puparium: for example, hormone-like substances, like Acps, produced in the paragonia, modify the transcription of genes related to muscle development, such as the gene encoding Muscle LiM protein or coracle (Heifetz and Wolfner, 2004; Kapelnikov et al., 2008). Such changes, and an effect on long-term mating memory, have also been observed in related diptera such as *Apis mellifera* (Kocher et al., , 2008).

I. 3.4The connection between gonads and genital disc

It is logical to think that, since gonads and genital disc are apart a long distance, from A8 to A5, there may be a signal coming from the gonads indicating the path that the derivatives of the genital disc should follow during its evagination. The first researchers entertaining that hypothesis were Stern and Hadorn (1939) who wondered if the directional migration was an autonomous effect or if it was due to a diffusible substance from the gonads. Their experiments showed that only when there was a contact between vas deferens, a structure derived from the genital disc, and testis, these would coil and colour, which would indicate a signal from the genital disc to the gonad, but not from the gonads towards the genital disc. They also described that vas deferens was covered by an "epithelium" of polygonal cells coming from the testis.

Babcock tried to find in females the diffusible substance inducing genital disc migration, she believed that gonads would produce the chemoattractant, and for that reason she performed ablation and transplantation experiments. In the absence of chirurgically removed ovaries, the discs would evaginate normally; however, when extra ovaries were introduced, free unattached oviducts were found in close presence of ovaries, what did not support the hypothesis of ovaries releasing a disc chemoattractant (Babcock 1971a; Babcock 1971b; Babcock 1971d) (Fig. I. 6 A). Hence, the question of how the genital disc would evaginate in the correct direction remained unanswered. The latest attempt to find a chemoattractant from gonads to genital disc pointed out to the protein Wnt2, which was present in male genital disc and gonads and whose absence resulted in uncoiled testes (Kozopas et al., 1998) Was this, then, a sex dependent process?

Earlier in the 30's some authors used chirurgical methods (ablating and implanting gonads and genital discs) and gynandromorphs production to know how the contact was made. Their main aim was to elucidate if there was a co-ordination between the genital disc and the gonads in each sex, or if male or female gonads would attract equally the genital discs independently of sex. Dobzhansky (1930) used gynandromorphs and observed that male gonads that contact female genital discs would degenerate (Fig. I. 6 C) while female gonads would develop essentially normally or smaller in size when attached to a male genital disc (Fig. I. 6 B). Transplantation experiments by Bodenstein (1946) confirmed these results and pointed out that for the testes to coil, they need to contact the vas deferens (Fig. I. 6 C), in disagreement with a previous report (Dobzhansky 1930), which hypothesized that gonads would develop and coil normally without contacting the genital disc.

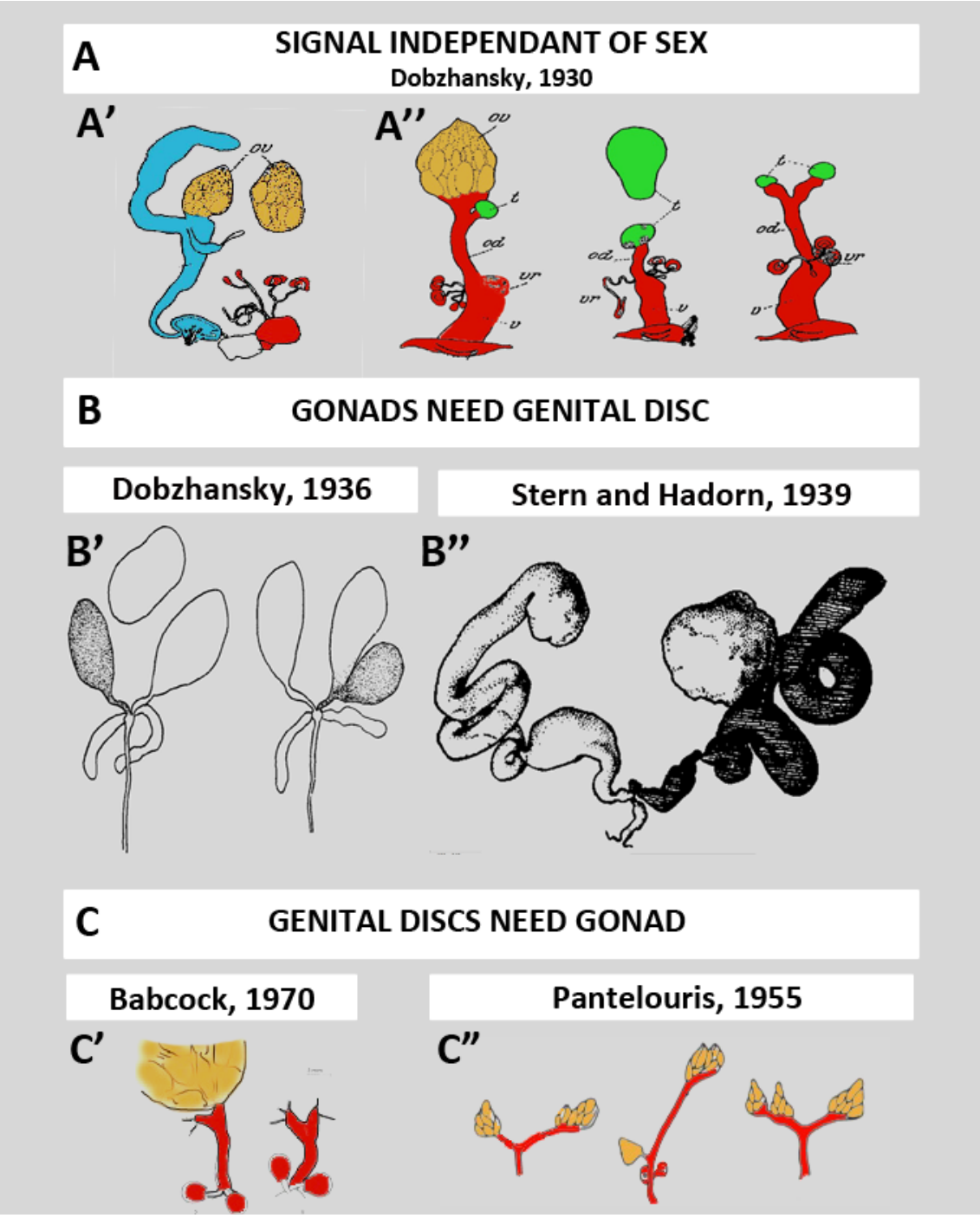


Fig. 6 Experiments studying the connection between genital disc and gonads to determine if signal is independent of sex.

A. Experiments with gynandromorphs. While ovaries (in orange) develop normally in contact with male structures (in blue) (A'), testes (in green) do no coil and seem to be degenerated when contacting female genital derivatives (in red) (A") (Dobzhansky 1930). B. Experiments with inter-species transplantation of extra male gonads. B'. While Dobzhansky (1936) postulated that gonads will develop normally independently of the contact with the genital disc, see the transplanted unattached white gonad with similar shape to the attached white gonad, compare to untransplanted darker gonad. B''., Stern and Hadorn (1939) described that for the testis to coil it was needed a contact with a coiling species genital disc. Compare the transplanted white gonad that belongs to an uncoiled species to the dark one, which is attached to its genital disc. Both of them are coiled. C. Experiments of gonad ablation and transplantation demonstrated that if oviducts do not find any ovary they present filamentous structures of unknown origin (C')

(Babcock, 1970). Oviducts were able to elongate to reach a distant ovary (C", Pantelouris, 1955). Dobzhansky supported this latter hypothesis in his experiments with gynandromorphs, in which he observed that even when there were gonads of both sexes in the same animal, there was no interference with normal development: the genital disc and gonads were able to contact the gonads independently of sex (Fig. I. 6 A). However, as separation of male and female tissue in gynandromorphs is at random, and since Dobzhansky (1930) could not effectively mark the sex of each cell, his results need further revision. Pantelouris, (1955) also made transplantation experiments where he showed that ovary growth does not depend on oviducts; rather the contrary, as oviducts may lengthen to attach an ovary located more distantly than the normal ones (Fig. I. 6 C), however, vas deferens do not depend on testis to develop (Fig. I. 6 A and B). In contrast with these results, Babcock (Babcock 1971b; Babcock 1971a) observed that oviduct elongation does not depend on ovaries, and that oviducts that fail to contact ovaries show a different morphology: the epithelium is thinner than in the wild type, muscle fibers appear disorganised and a filamentous structure may be observed at the tip of the oviduct. Although the nature of this structure was unspecified, she proposed it to be of tracheal or nervous nature. It was also observed that extra female genital discs transplanted into a male larva would be underdeveloped and showed fewer muscles.(Bodenstein 1946). More recently, Kozopas et al. (1998) proposed Wnt2 as a possible chemoattractant candidate, but failed to explain why it is present both in gonads and genital disc.

I. 4 Estructure and Coiling of the testes

As mentioned before, elongation and coiling of the testes takes place during pupation, after the ecdysone pulse that triggers metamorphosis. Before pupation, larval testes are of ovoid shape and they show an outer layer of pigment cells (described in more detail below) that surrounds and protects four different population of cells: a) in the apical area, the stem cells coming from of the germ line; b) a small group of cells forming the hub, a structure formed by a specific type of gonadal somatic cell that allow the maintenance of stem cells status of germ cell derived (Germinal Stem Cells, GSCs) and somatic cell derived (Cyst Stem Cells, CySCs) cells; c) at the base, a specific type of cells, msSPGs; d) the vast majority of cells in the gonad, Somatic Gonadal Cells (SGCs) which form the supporting tissue that will provide the information needed by GSCs during their differentiation (Jemc, 2011). In the adult, there is a new layer of tissue, the muscle, located between pigment cells and the other four testis cell populations but excluding the hub, as muscle is discontinued in the stem niche (Fig. I. 4 D, Hardy et al., 1979).

Muscle precursors are not present in the gonads prior to the contact (Kozopas et al., 1998). Hence, muscles must come from the genital disc. The contact between gonad and genital disc is produced 24h APF and it leads to elongation and coiling of the testes (Fig. I. 4 D). However, it is important to note that there is a double migration after the contact: while muscles from the genital disc migrate under the PC to surround the gonad, pigment cells from the testes migrate over the muscle to coat the vas deferens. Therefore, the adult testis presents three layers of tissue, which from the outside to the inside are: pigment cells, muscle, and the same four cell populations (Fig. I. 4 C), while, vas deferens epithelium is covered by pigment cells, a sheet of muscle and the epithelium.

Different species of *Drosophila* show different degrees of coiling. *Drosophila melanogaster* testes coil two and a half loops (Fig. I. 7 B), *D pseudoscura* half a loop (Fig. I. 7 A) and D. *virilis* more than four loops (Fig. I. 7 C). Testes length is related to sperm length and, therefore, the longer the sperm, the longer the testis (Joly and Schiffer, 2010). Besides, this coiling always takes place in the same direction in each testis, which has been taken as a model of left-right asymmetry (Coutelis et al. 2008).

In the process of reproductive system development, after the contact between gonads and vas deferens, there is a ten-fold increase of volume, which pushes the coiling of testes (Fig. I. 7). The coiling might be due to the differential excretion of membrane from left and right sides of the testis (Miller, 1941). However, the mechanism behind the coiling is not yet known.

In what follows, we will introduce the two tissues forming the testis sheath, muscles and pigment cells, describing how they originate and how do they communicate between them, in order to understand the process of coiling. Whether coiling is required for fertility was first addressed by Woolf (Woolf 1965; Woolf 1968), who considered that morphology was not related to fertility, as he observed motile sperm in unattached testis. However, he did not perform fertility assays so the question of fertility in these cases remains open.

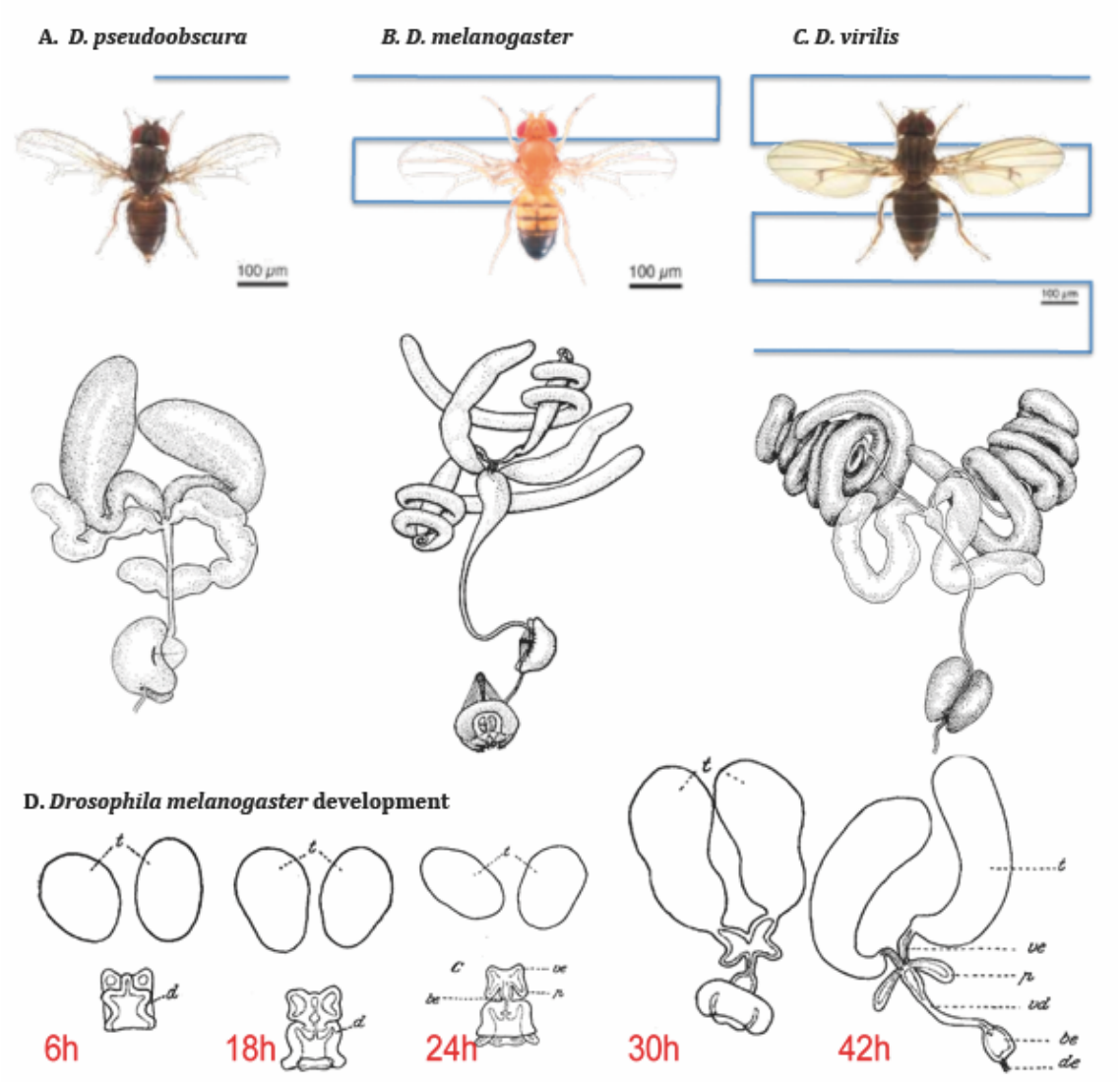


Fig. 7 The relationship between coiling and sperm length.
Comparison of three different species of Drosophila testis and spermatozoid length. At the top, male adults of the each fly and in blue a scaled spermatozoid. At the bottom, a graphic of the internal reproductive system, showing different degrees of coiling (From Patterson, 1941). A. D. pseudoscura has a 263µm spermatozoid and its 867µm-long testes do not coil (Joly and Bressac, 1993). B. D. melanogaster has a 1910µm spermatozoid and testes of 1880µm and 2.5 loops (Pitnick, 1995). C. D. virilis, showing a 6470µm 4-time looped spermatozoid and 5600µm-long testes (Joly and Bressac, 1993). D. Progressive elongation and coiling of the testis after the contact with genital disc derivatives in Drosophila melanogaster (modified from Epper, 1983a).

I. 4.1Pigment cells

Pigment cells (PC), together with muscle cells, form the outer sheet of the adult testis (Fig. I. 4 C) These cells of mesodermal origin are only present in males and their name is related to their being responsible for the change of colour in the testis and vas deferens in *Drosophila* males after emerging (Bairati, 1967). PC colouring has been related to an intra- and inter-species signal allowing females to tell apart male fertility and discriminate males

from different species of Drosophila too (Markow and O'Grady, 2006, Fig. I. 8 A). PC have a polygonal shape and present a big nucleus, which indicate that they are most probably polytene cells (Bairati 1967; Fig. I. 8 C and C').

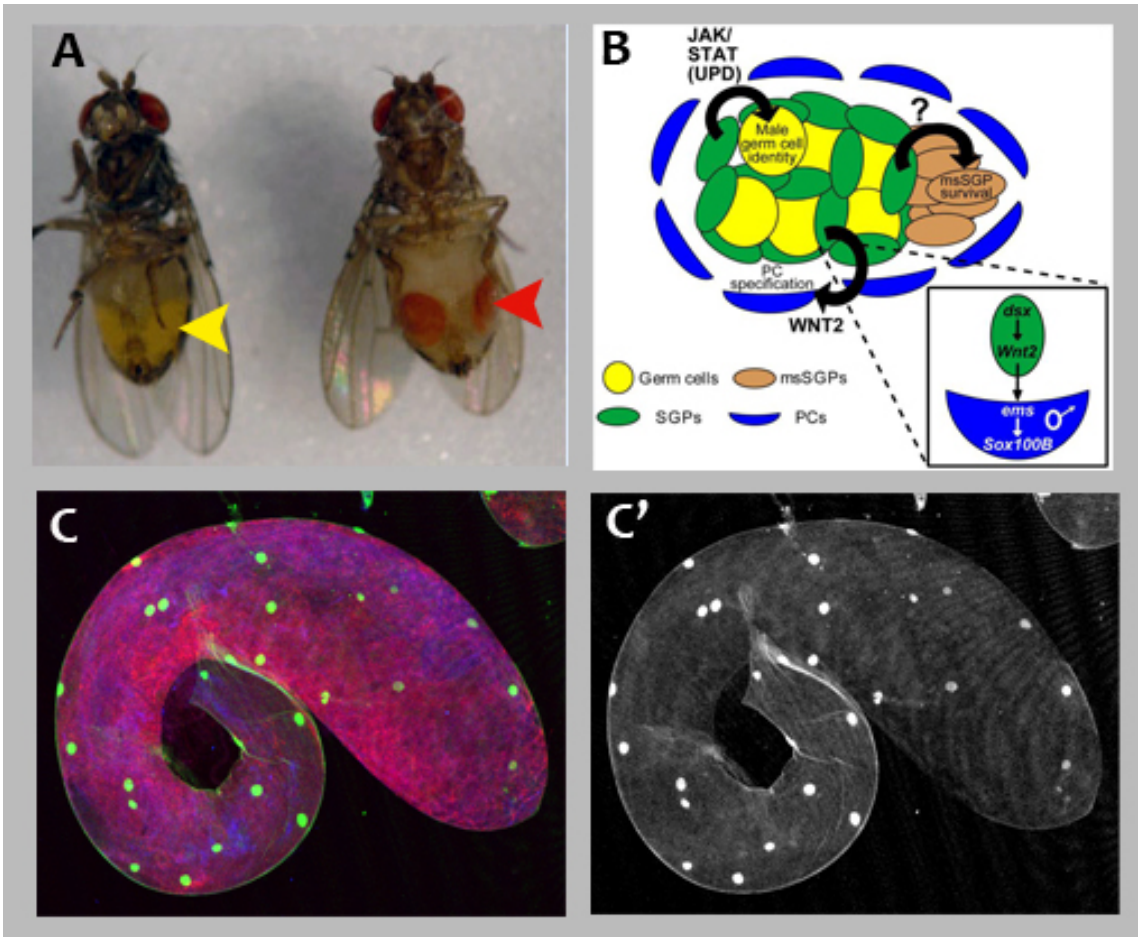


Fig. 8 Description of Pigment cells.
A. Different species of Drosophila have different colours of pigment cells, which can be observed through the abdomen; the arrows point the testis and match their colour (Markow and O'Grady, 2006). B. Pigment cell specification in the embryo (DeFalco et al., 2008) C. SOX100B-Gal4 UAS-GFP testis where pigment cells nuclei are seen in green.

These cells differentiate from a small population of cells of the fat body that express *Krüppel* and that receive the Wnt2 ligand secreted by male somatic gonadal cells (SGCs). Wnt2 is produced only in male gonads and it induces the expression of *empty spiracles* (*ems*) in pigment cells, which in turn activates *SOX100B* (Fig. I. 8 B) (DeFalco, et al., 2008; Nanda et al., 2009). Previous studies have shown that mutants of *Wnt2* (Kozopas et al., 1998) and *SOX100B* (Nanda et al., 2009) lack pigment cells. While the former study demonstrated that *Wnt2* is both necessary and sufficient to induce the differentiation of PCs, it could not explain their function. They hypothesize that Wnt2 could be the gonadal signal attracting the genital disc, or else, be required for the correct determination or migration of muscles, as they observed that the muscle layer was malformed. Additionally,

DeFalco et al., (2008) demonstrated that *SOX100B* is active in the msSPGs and pigment cells, though it was not sufficient to induce the formation of PCs. They observed that while the gene was not required for the survival of the germ line, *SOX100B* mutants had no PC and, moreover, presented fat body histolysis problems, which may account for the pupal lethality of *SOX100B* mutants (Nanda et al., 2009). Interestingly, these mutants showed variable reduced testicles with abnormal morphology, sometimes even missing, although gametes seemed unaffected. Female gonads also showed disorganized connective and tracheal tissues. They conclude that the lack of PC was due to their degeneration during pupal stages.

I. 4.2The muscle layer of the testis

Muscles are contractile tissues derived from the mesoderm. The mesoderm gives rise to at least three different types of muscle: cardiac, visceral and somatic muscle; it also forms the fat body from which pigment cells, hemocytes and lamelocytes derive. All the muscles are determined early in the embryo and their fate depends on three degrees of specification: autonomous, segmental and non-autonomous identity. The latter depends on adjacent or nearby tissues that may impact muscle determination, such as nervous tissue defining, for example, the Muscle of Lawrence (Lawrence and Johnston, 1986). Segmental identity of the muscles relies on epidermis information, and in this sense, might be considered also an non-autonomous determination (Dutta et al., 2004; Dutta & VijayRaghavan, 2006). Finally, autonomous muscle identity is defined by a specific set of genes called elector genes that may be transcribed at the same time or in a finely orchestrated fashion (Joussineau et al., 2012; Dutta and VijayRaghavan, 2006). This autonomous definition has been thoroughly studied mainly in indirect flight and jumping muscles; however, little is known regarding the reproductive system muscles.

As said before, the segmental identity is defined by the segmental position of precursors determined by segmental polarity genes and maybe Hox genes too. In this sense, we would expect that genes related to the definition of the posterior part of the body may be needed in the specification of the reproductive system muscles, as they are located in this posterior region. Up to date, only one study (Broadie and Bate, 1991) has addressed that question and found that activation of *Spitz* by *rhomboid* would in turn activate *lethal of scute* (*lsc*) responsible for posterior muscle determination.

Regarding the autonomous definition of muscles, it takes place already in the embryo, when different levels of Notch determine by asymmetric divisions two different populations from the original pool of myoblasts. Those two populations are founder cells

(FCs), closer to the ectoderm, and fusion competent myoblasts (FCMs), closer to the endoderm. The founder muscles cells are defined by the transcription of elector genes such as *Krüppel* (*Kr*), *even skipped* (*eve*), *Nautilus* (*nau*), *ladybird* (*lb*) or *Drop* (*Dr*), also known as *muscle homeotic gene* (*msh*); fusion-competent myoblasts need *gleeful* (*glee*) or *lameduck* (*lmd*). The fusion process requires *stick-and-stones* (*sns*) and *Hibris* (*Hbs*) in the FCs and *blownfuse* (*blow*), *dumfounded* (*duf*) and *roughest* (*rst*) in the FCMs, and later on *Deliah* (*Dei*) for the attachment (Dobi et al., 2011) (Fig. I. 9 A).

The muscles defined at embryonic stages divide at the larval period to form the larval muscles. At the beginning, all the divisions are synchronic and later on the divisions occur first in the ventral, then in the lateral and last in the pleural muscles. Since there is little, if any, exchange among muscle populations, it can be assumed that all muscles are defined at the embryo stages and have a unique identity (Broadie and Bate, 1991). Some of the founder cells remain dormant until metamorphosis when the ecdysone pulse resumes their development, thus forming the adult muscles. Adult muscle differentiation depends first on Mef2 and Myosin and later on Kettin, which allows contractility (Chen et al., 2007) but attachment and binding to tendons also have an impact on muscle aspect and function.

Traditionally, muscle type was defined by its aspect. This is based on the presence or absence of sarcomere, which is the functional and anatomic unit of the striated muscles. The basic unit of striated muscle is the sarcomere, it is defined as the region comprised between two Z discs o Z-lines (Zwischenscheibe) composed of Kettin, and which are seen as denser bodies at the electronic microscope. The Z discs anchor two different types of myofilaments: thick myofilaments of myosin and thin actin filaments (Fig. I. 9 B and B'). The different light properties of actin and myosin and their organisation allow to recognise three bands: a) isotropic I-band, made of thin actin filaments and closer to the Z-disc; b) the anisotropic A-band, whose length corresponds to thick myosin filaments length and in which the H band may be observed, where myosin and actin do not overlap; and finally, c) the M line, which may be observed at the H band and it is also denser to electrons due to the linkage of cytoskeleton elements (Fig. I. 9 B"). In the absence of the sarcomere , we could consider a muscle as non-striated or smooth.

In the reproductive system of Drosophila, the muscles have been classified as: striated (dark green) or smooth (light green) and mononucleated (dark blue) or multinucleated (light blue) (Susic-Jung et al., 2012) (Fig, I. 9 C). Therefore, we can recognise three types of muscles: striated mononucleated in the ejaculatory duct and paragonies, striated multinucleated in the spermatic pump and the vas deferens and finally, the unique smooth multinucleated muscle of the testis. Apart from the fibber organization, little is known

regarding the autonomous genetic identity of the reproductive system muscles. Taking into account that the microenvironment may affect the cell fate of, for example, the stem cells and their niche (Gattazzo et al., 2014; Guilak et al., 2009), we would like to summarize here the importance of the micro-environment and particularly the role of the Extra Cellular Matrix in our system.

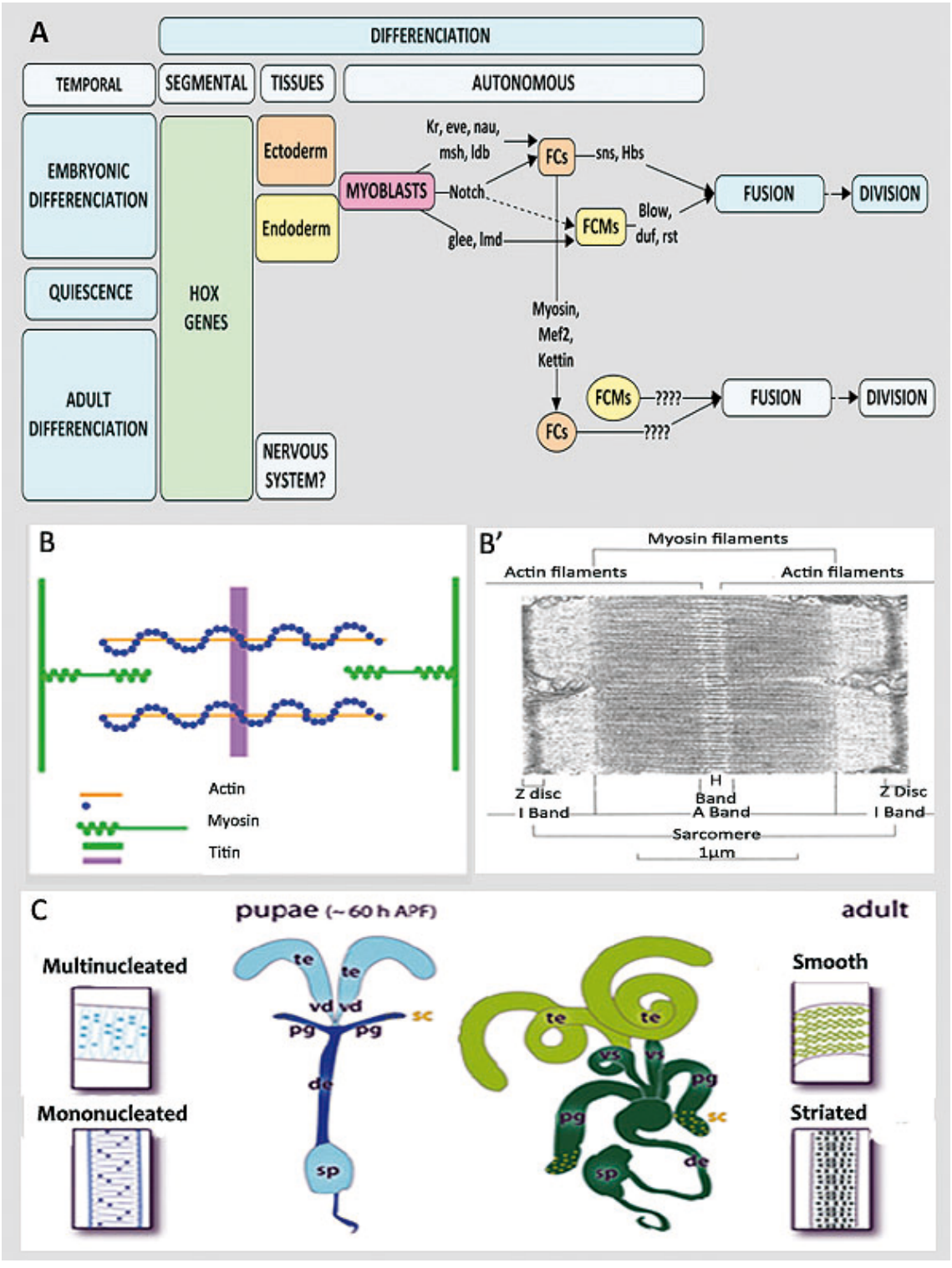


Fig. 9 Muscles types based on morphology and their differentiation.
A. Muscle cell specification and development. Segmental, tissue and autonomous specification during the development of Drosophila melanogaster. After the specification, FMCs and FCs fuse to give rise to the muscles, which will then divide to grow (Broadie and Bate, 1991). B. Basic structure of the sarcomere: filamentous actin in orange, glomerular actin in blue, myosin in green and Titin in purple. Differences in those proteins are related to muscular differences and properties..B'. Organization of bands based on optical properties. C. Muscles types in the reproductive system of Drosophila, as defined by (Susic-Jung et al., 2012)

I. 4.3Extra-Cellular Matrix in *Drosophila*.

Every tissue is embedded in a matrix of proteins and gluco-polysaccharides, known as extra-cellular matrix (ECM). Apart from being a scaffold (Basteck and Johnson, 2011) it also provides positional information essential to differentiation and tissue polarity. Most of the cells are polarized, with apical and basal sides. This polarization is achieved by the differential localization of proteins and some other cellular membrane components which allows carrying out different functions within a cell, e.g. to exchange material in the apical surface, communicating and signalling with neighbouring cells on the lateral sides, and to receive spatial information from the basal lamina (Bryant and Mostov, 2008).

The information provided by the ECM allows the establishment of the microenvironment, which is of vital importance to maintain stem cell population as it provides the right niche for their development (Papagiannouli et al., 2014). The impact of the microenvironment in cell fate has also been shown in epithelia where it may introduce changes during morphogenesis (Bryant and Mostov, 2008; Srivastava et al., , 2007) and may also impact organ shape (He and Adler, 2002; L. He et al., 2010; Gates, 2012; Horne-Badovinac, 2014; Pastor-Pareja and Xu, 2011). Since the ECM connects different tissues, it also helps transmitting information between two of them (Wang et al., 2008) and communicates the outside of the cell with the inside (Pinheiro and Montell, 2004; Cerezo et al., 2009), serving also as a barrier for metastasis (Goetz et al. 2011; Srivastava et al. 2007). The role of ECM has not been studied in the reproductive system of Drosophila. However, it is possible that it is involved in signalling between different cell layers in the testes. It may also have a role in Left/Right asymmetry as some of its components, such as the orthologous to Laminin ß1 are related to the establishment of laterality in the organs in vertebrates (Gates, 2012; Hochgreb-Hägele et al., 2013).

I. 5 Cell signalling

Since the main tissues forming the testis have been described, we would briefly report signalling in their cells. As mentioned before, there is a basic set of genes and mechanisms

shared by all the living forms. One of those mechanisms is cell communication, mediated by gene cascades that allow the translation of outside information into transcriptional response in the nucleus (Fig. I. 10). There are several conserved signalling pathways in Drosophila and vertebrates: Dpp/TGFß, Wg/Wnt, Notch, Hh/Shh, etc. One of them is the Jun Kinase Pathway, known as JNK pathway, and which is related to "distant" processes such as cell migration, wound healing or apoptosis, most of them related to cellular adhesion (Srivastava et al., 2007). Other pathways are already known to be needed in some aspects of gonad or germ line development. For example, Notch aids to discriminate founder cells from fusion competent myoblasts during muscles development, formation of the gonads requires the activation of the JAK/STAT pathway for the stem-cell maintenance and later development, and the Insulin/Tor pathway is active on follicle cells determining size related to the nutrients availability (Barth, 2011). However, nothing is known about the possible role of different pathways in testis development at pupal stages. We are also interested in knowing the integration network of these signalling pathways as it may shed light to the question of how the different structures in the reproductive system communicate and coordinate their information to shape the testes and give rise to a fertile individual.

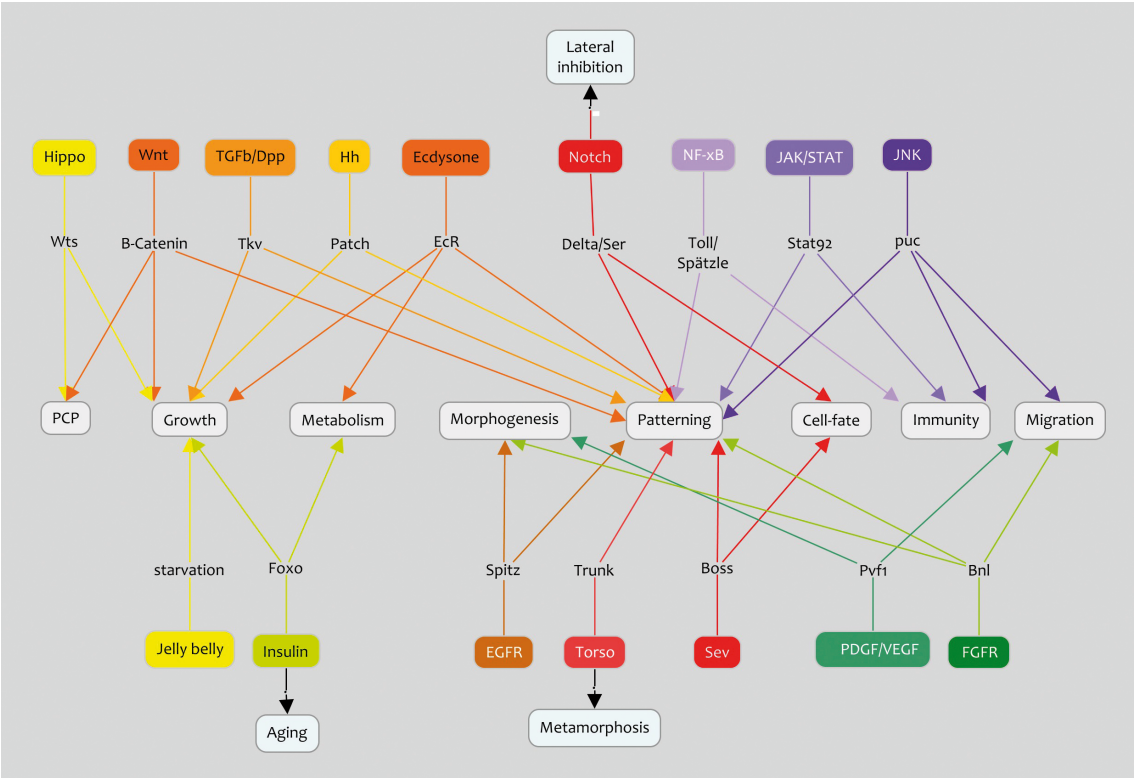


Fig. 10 Diagram of different signalling pathways (in colours)and the processes in which they are involved (in white)

OBJECTIVES

1. To determine the mutual need between gonads and genital disc for their proper development during pupa, that is, if the gonads are needed for the genital disc to evaginate and to form the reproductive system and if the genital disc is required for the gonads to develop normally.
2. To study how the coiling of the testes is produced and which are the tissues and signalling pathways implicated in this process.
3. To investigate the determination of testis smooth muscle and if signals, coming from Pigment Cells and the Extra Cellular Matrix, may alter its identity.
4. To examine the relationship between the process mentioned above and fertility.

MATERIALS AND METHODS

M. 1. Fly stocks

M. 1. 1. Mutants

abd-A^{M1} is a null allele of *abd-A* (Sánchez-Herrero, et al., 1985); *iab-4*^{302} is a mutation that affects the expression of *abd-A* and which causes transformation of A4 into A3 (Karch et al., 1985). *Uab*^{1} is a null allele for AbdB-R and *Abd-B*^{M1} is a mutant defective in the activity of isoforms AbdB-R and AbdB-M (Casanova et al., 1986; Sánchez-Herrero and Crosby, 1988; Foronda, 2007). *cad*^{2} (Moreno and Morata, 1999) and *cad*^{3,} (Wu and Lengyel, 1998) are *cad* null mutants (*byn*^{5} is a *byn* null mutant (Shinmyo et al., 2006). The combination *Df(3R)j3B9-rv12* / *Df(3R)tll-g* is deficient for *sox100B* (Nanda et al., 2009) and the combination *DWnt-2RJ/Df(2R)11*, for *D-Wnt-2* (Kozopas et al., 1998).

M. 1. 2. P-lacZ lines

P-lacZ is a transposable element in which ß-galactosidase expression allows to detect the activity of the regulatory sequences near the point of insertion of the transposon. We have used the following P-lacZ lines: for muscles, *ladybird early-lacZ (lbe-lacZ*) and *ladybird late-lacZ (lbl-lacZ*) (De Graeve et al., 2012); to detect Notch pathway activity, *Enhancer of split*-lacZ (*E(spl)*-lacZ) (Cooper et al. 2000) and *big brain*-lacZ (*bib*-lacZ) (Rao et al. 1992), for the JNK-pathway, *puckered-lacZ (puc-lacZ*) (Martín-Blanco et al., 1998), and for the JAK-STAT pathway, *hopscotch*-lacZ (*hop-lacZ*) (Kania et al., 1995), and *Ken and Barbie-lacZ* (*ken*-lacZ) (Castrillon et al., 1993). Notch activation was also detected by the *E(spl)mß-CD*2 reporter (Celis et al. 1998) that we include here although it is not a P-lacZ line.

M. 1. 3. GFP-trap lines

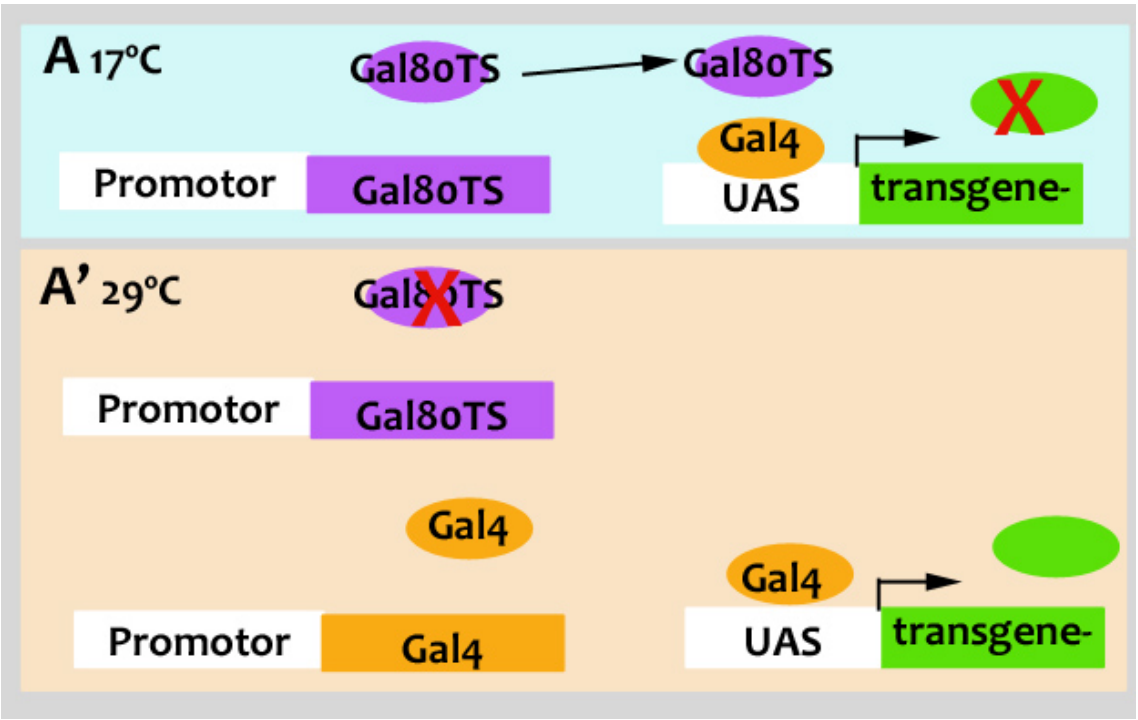
We have used the following Protein-trap lines to ascertain the expression of different proteins. In the muscle, the position of the Z-disc was determined by the presence of TITIN (Titin-GFP;, Hudson et al., 2009), and Integrin Linked Kinase ILK (Ilk-GFP; Zervas et al., 2001). The activation of the JAK/STAT pathway was ascertained by Signal-transducer and activator of transcription protein at 92E STAT92-10 x GFP (Bach et al., 2007).

M. 1. 4. The Gal4/UAS system

The Gal4/UAS technique allows to control the expression of a chosen gene (cDNA), which is under the control of Upstream Activator Sequences, UAS. The temporal and spatial control of gene expression depends upon the presence of the Gal4 protein, that responds to specific enhancers that vary for each line (Brand and Perrimon, 1993). It is possible to prevent Gal4 activity by driving ubiquitous expression of the Gal80 protein, *tub*-Gal80 (Lee and Luo, 1999), which inactivates the Gal4 protein, blocking therefore the Gal4-UAS system. A specific type of Gal80 protein, Gal80^{ts}, is termolabile; it allows the degradation of the Gal80 protein and the activation of Gal4 at 29ºC but it prevents Gal4 activity at 17ºC (McGuire et al., 2003) (Fig. M1).

M. 1. 4. 1. Gal4 lines

In this thesis we have used different Gal4 lines to specifically direct gene expression in gonads, genital disc, pigment cells and muscles. Some of the Gal4 lines and most of the UAS lines (see below) were obtained from the Drosophila Stock Centers in Bloomington (USA) and Vienna. *cad*-Gal4^{EM469} and *cad*-Gal4^{MD509} drive expression of Gal4 in the area of the genital disc giving rise to the analia and hindgut (Calleja et al., 1996) (Moreno and Morata, 1999).



Gal4/Gal80ts system

A. Scheme of the activity of Gal80 termosensitivity at 17ºC, where it is active, and at 29ºC, where it is not and therefore allows the transcription of Gal4, which directs the expression of the transgene (A').

c855a-Gal4 (Bloomington 6990; Hrdlicka et al., 2002), and *1151*-Gal4 (myoblast Gal4-line), obtained from L. S. Shashidhara (Centre for Cellular and Molecular Biology, Hyderabad, India; (Roy and Raghavan, 1997)) were used to drive expression in the muscles of the vas deferens; *MJ12a-Gal4* (Hrdlicka et al., 2002) and *SOX-100B-Gal4* (Bloomington 27779, Nanda et al., 2009) to drive expression in the PC, *He*-Gal4, (Pastor-Pareja and Xu, 2011) directs expression in the fat body and hemocytes, and *c355*-Gal4 directs broad genital disc expression (Gilboa and Lehmann, 2006).

M. 1. 4. 2. UAS lines

To report the activity of each Gal4 line driver we have used UAS-GFP (Bloomington stock 1522), which expresses GFP in the nucleus and UAS-CD8RFP (Bloomington stock 27391, Frank Schnorrer which expresses CherryRFP fused to the mouse CD8 extracellular and transmembrane domains under UAS control; the UAS-*hid* and UAS-*rpr* lines were used to ablate cells and are described in Flybase.

M. 1. 4. 2. 1. UAS-RNAis lines

We have used many different RNAi lines to study the effect of reducing gene expression in different tissues and organs. The name of the line, the provider and number of off-targets, if any, are stated. When the provider did not state the number of off-targets, we performed an off-target assay with the program described on the webpage http://dscheck.rnai.jp/. We have also included in this heading some UAS lines that are not UAS-RNAi lines but that inactivate also different genes.

We used different RNAis against genes expressed in muscles: UAS-*Dr* RNAi (Bloomington 26224), UAS-*Dr* RNAi (Vienna 110625, 4 off-targets); UAS-*Six4* RNAi (Bloomington 30510), UAS-*bin* RNAi (Bloomington 34718), UAS-*Alk* RNAi (Bloomington 27518), UAS-*Mef2* RNAi (Vienna 28699) and UAS-*Abd-B* RNAi (Vienna 104872). To inhibit cell-division and prevent muscle migration we used UAS-*cdc2 RNAi* (Vienna 41838).

To study pigment cells determination we have targeted *SOX100B* with UAS-*SOX100B* RNAi (Vienna 45961), *Wnt2* with UAS-*Wnt2* RNAi (Bloomington 28892 and Bloomington 29441), and *ems* with UAS-*ems* RNAi (Bloomington 28726). For the studies of the hindgut we have used UAS-*cad* RNAi (Bloomington 34702) and UAS-*byn* RNAi (Vienna 108984 and Vienna 1015349).

To determine the involvement of the different signalling pathways in our studies we have used UAS-*Bd* RNAi (Vienna 27174) to target Notch receptor and UAS-*E(spl)* RNAi (II) to inhibit the Notch activation. The JNK pathway was inhibited using UAS-Bsk^{DN} (Adachi-Yamada et al., 1999), UAS-*puc14C* (Martín-Blanco et al., 1998), UAS-*puc2A* (Colombani et

al., 2012) and we have used UAS-*mmp1* RNAi (Bloomington 31489) to prevent cell migration triggered by JNK. To inactivate the JAK/STAT pathway we have used UAS-*STAT92E* RNAi (Bloomington 33637), UAS-$dome^{DN}$ (Bloomington 32860 and Bloomington 34618), UAS-*hop* RNAi (Bloomington 32966), UAS-*upd2* RNAi (Bloomington 33949) and UAS-*upd3* RNAi (Bloomington 32859).

To change the sex (or eliminate sex determination) we have used UAS-*dsx* RNAi (Vienna 110306, 6 off-targets and Bloomington 35645), UAS-*tra* RNAi (Bloomington 28512) and UAS-*tra* (Bloomington 4590). To study the role of the extracellular matrix (ECM) in the development of the reproductive system we have used RNAis to target the different genes: on the outside of the cell we have targeted Drosophila proteoglycan Trol, UAS-*trol* RNAi (Vienna 24549 and Bloomington 29940) and UAS-*lamA* RNAi (Vienna18873. In muscles, these proteins are linked to Thrombospondin, and we have also used a UAS-*Tsp* RNAi (Bloomington 29399). Laminins on the surface are linked to Dystroglycan in muscles and integrins in every cell. We have used UAS-*Dg* RNAi (Bloomington 34895) and expressed RNAis against the integrin different subunits: the α−subunits, Inflated (UAS-*If* RNAi) and mew (UAS-*mew* RNAi) and ß-subunit or Myospheroid (UAS-*ßPS* RNAi, Bloomington 27735 and UAS-*Mys* RNAi, Bloomington 33642). We have also targeted integrin linked proteins in the inside of the cell such as Ilk (UAS-*Ilk* RNAi Bloomington 35374), Tallin ¿talin? (UAS-*rhea* RNAi, Bloomington 33913) and the focal adhesion kinase Fak (UAS-*fak* RNAi, Bloomington 29323), but also in the outside of the cell such as Tiggrin (UAS-*Tig* RNAi, Bloomington 31570)

M. 2. Clonal analysis

To obtain clones mutant for the *iab-4* regulatory region of *abd-A* we first made the FRT82B $iab\text{-}4^{302}$ recombinant. Clones were induced in second or early third instar larvae of the genotype *y w* hs-*flp1.22*; FRT82B *Ubi*-GFP / FRT82B $iab\text{-}4^{302}$, grown at 25ºC, with a 1h heat shock at 37ºC, and kept at 25º until wandering larvae were seen and dissected.

M. 3. Immunohistochemistry

We performed immunochemistry assays in larvae, pupae, pharates and adults. They were made as previously described (Sullivan et al., 2000), with slight modifications, following this protocol:

Larvae, pupae and adults were dissected in PBS 1X and transferred for fixation to a cold solution of 4% paraformaldehyde + 0.1%Triton, 1%DOC. They were incubated at room

temperature (RT) for 27 min or at 4ºC overnight, then rinsed 4 times with washing buffer (PBS 1x, cow serum, triton X-100) and incubated with primary antibodies at 4ºC overnight. After that, they were rinsed twice with washing buffer (PBS 1x, cow serum, tritonX-100 and washed 3 times for 10 min with washing buffer. We incubated them with secondary antibodies 2h at RT and then rinsed twice with washing buffer and washed 3 times for 10 min with washing buffer. Finally, they were transferred to Vectashield™ mounting media.

The antibodies used are described below.

M. 3. 1. Primary antibodies

Mouse anti-Hu li tai shao (anti-Hts) (1B1, 1:20; from the Developmental Studies Hybridoma Bank (DSHB)) (Davis and MacIntyre, 1988; Yue and Spradling, 1992; Ding et al., 1993), mouse anti-Abd-B (lA2E9, 1:20, DSHB, Celniker et al., 1989), mouse anti-Crumbs (1:20) from DSHB, mouse anti-Evenkipped (anti-Eve) (1:20) from DSHB, mouse anti-Eya (1:20) from DSHB (Bonini et al., 1993), mouse anti-Mmp1 (1:100, larvae; 1:20, pupae and adults) from DSHB, mouse anti-Wg (1:20) from DSHB, mouse anti-Notch intra (1:20) from DSHB, mouse anti-Notch extra (1:20) from DSHB, mouse anti- Integrin α Subunit (multiple edematous wing) (anti-PS1) (1:20) from DSHB, mouse anti-Integrin α Subunit (Inflated) (anti-PS2), (1:20) from DSHB, mouse anti-Integrin ß Subunit (anti-ßPS) (1:20) from DSHB, mouse anti-Vkg (1:20) from DSHB, mouse anti-ß-galactosidase (Cappel), rabbit anti- ß-galactosidase (1:2000) from Cappel, rabbit anti-LamB1 (1:20), rabbit anti-PH3, 1:400 (Cell Signaling Technology), rat anti-AbdA (1:100) (MacÍas et al., 1990), rat anti-Ems (1:100) (Macías and Morata, 1996), rat anti-Tropomyosin (MAC141; 1:100; Babraham Tech.) rabbit anti-HRP (Jan and Jan, 1982), rat anti-NC82 (1:20), from DSHB.

M. 3. 2. Conjugated secondary antibodies

The following antibodies were used at 1:200 dilution: anti-rat, anti-mouse and anti-rabbit conjugated with fluorochromes Red-X, Texas Red, FITC y Cy5 (Jackson Immunoresearch), To-Pro-3 (Invitrogen) .

M. 4. Fertility tests

Males were mated individually with three Canton-S newly eclosed females in a single vial, kept for three days at 25 ºC and the number and viability of eggs checked. We assessed male fertility based on the number of viable eggs laid by females. When viable eggs were found, males were considered fully fertile, while when those eggs did not hatch into larvae,

we considered the males sterile. Individual males were dissected to check the status of the reproductive system and noted as: pigment cells defective, when the testis did not show the wildtype yellow colour, morphology defective, when the testis did not show the wild-type elongated and coiled shape, or both, when males showed white deformed testes.

M. 5. Imaging

M. 5. 1. Microscopes

Fluorescently stained larvae, pupae and adults were mounted in Vectashield™ and visualized using one of the following available microscopes:

- Leica TCS SPE Confocal microscope
- Zeiss Confocal LSM510 META Confocal microscope
- Zeiss Confocal LSM510 Scanning Confocal microscope
- Zeiss Confocal LSM510 Inverted Confocal microscope

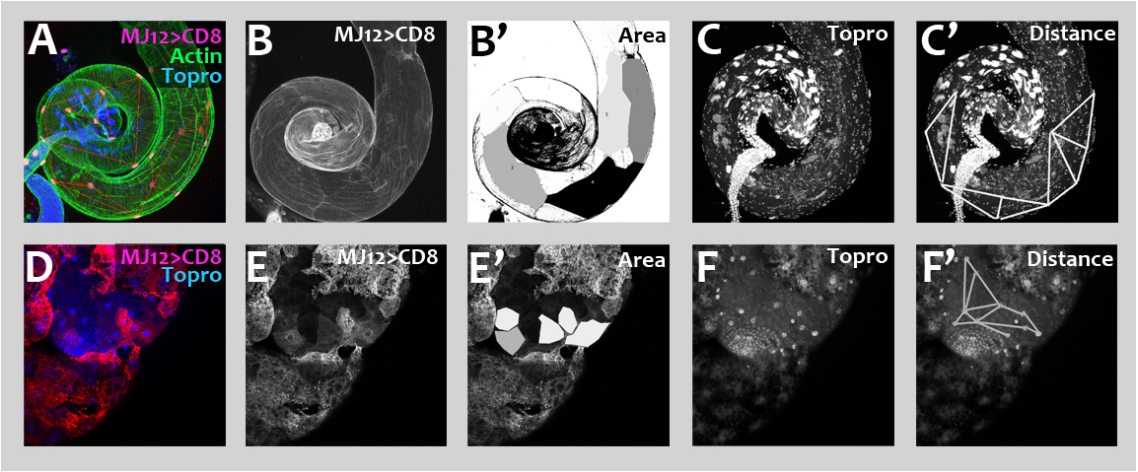
M. 5. 2. Images analysis and processing

Images were processed and analysed using ImageJ (NIH) and figures designed on Adobe Photoshop and Adobe Illustrator.

M. 5. 2. 1. Pigment Cells analysis.

To determine the number of PCs, the area and the distance among the nuclei, we used ImageJ (NIH). For each stack of images of a testis, we first determine the total area of testis (Fig. M. 2 A) and we examined the red channel (*MJ12a > CD8RFP,* Fig. M. 2 B) to determine PCs area (Fig. M. 2 B') and the blue channel (To-Pro 3, Fig. M. 2 C) where the PCs nuclei would be easily determined by their position (outer sheath of the testis) and size (big nuclei).

Areas and distances between nuclei were analysed with the "Measure" tool of Image J. To measure a distance we draw a line between the nuclei centres (Fig. M. 2 C and C') and to measure the PCs area, we draw a polygon following the cell-membrane (visible with *MJ12a > CD8RFP,* Fig. M. 2 B and B'*),* they were later coloured to avoid repetitions (Fig. M. 2 B'). We measure testis total area similarly to the PCs area, but they were not coloured after measurement. Nuclei were counted with the cell counter tool. After measurements, we calculate mean testis area, PCs number, area and distance among nuclei for each genotype.



Determinations of PCs number, area and distance

A-C' Adult testis. A. Total area of an adult testis and PCs number. B. Adult PCs area before analysis. B'. Adult PCs areas after analysis. C. Adult PCs number and distance among nuclei, before analysis. C'. Adult PCs number and distance among nuclei after analysis. D-F'. Larval testis. D. Total area of a larval testis. E. Larval PCs area before analysis. E' Larval PCs area after analysis. F. Larval PCs number and distance among nuclei before analysis. F'. Larval PCs number and distance among nuclei after analysis.

M. 6. Data analysis, graphics and layout

Data analysis was performed with Microsoft Excel data sheets, graphics were created with Excel and Adobe Photoshop, except for the diagrams and flowcharts, for which I used C-Maps tools. Figures were elaborated in Adobe Photoshop and Adobe Illustrator. General layout for printing was designed with Adobe InDesign.

RESULTS

R. 1 The contact between gonads and genital disc in the development of the reproductive system

During normal male and female pupal development the genital disc evaginates, elongates towards the anterior as it develops several structures, and contact the gonads to form the reproductive system. After the contact, some changes in male and female gonads take place, being most evident in testes. We wanted to identify the mechanisms required for such connection and also how this communication induces the elongation and coiling of the testes.

We will tackle first how gonads and genital disc contact each other. This question was addressed first in the 1930's and following decades using different chirurgical methods of ablation and transplantation and with gynandromorph analysis; however, the results did not solve the controversy between the need or not of gonads for genital disc pupal development (or vice versa). Some experiments demonstrated that in the absence of the gonads the genital disc would evaginate normally while others maintained that gonads were required for correct formation and elongation of the genital disc derivatives (Dobzhansky, 1930; Bodenstein, 1946; Babcock, 1971a; Babcock 1971b). Besides, these results were obtained mainly by surgical methods, which are sometimes not very precise and might not be as reproducible as those using genetic approaches. For these two reasons, to try to solve this question we have studied the development of the genital disc derivatives in mutants lacking gonads and of gonads in mutants with aberrant or no genital disc.

R. 1. 1 Lack of connection between gonads and genital disc

Some experiments demonstrated that when gonads where surgically removed the genital disc would evaginate in a normal fashion (Bodenstein, 1946; Pantelouris, 1955; Babcock, 1971a) (Fig. I. 6 A and C), but that the gonads would not fully develop in the absence of genital disc or when there was no contact between them (Dobzhansky, 1930). As stated above, we have studied these issues with a genetic approach.

R. 1. 1. 1 The genital disc evaginates correctly in mutants lacking gonads

To genetically ablate gonads we sought genes required for gonad formation; however, null mutations in most of them, like *tinman* or *six4* (Broihier et al., 1998; Moore et al., 1998; Kirby et al., 2001), were embryonic lethal, and therefore not suitable to answer our

question. However, the homeotic gene *abd-A* is expressed in gonadal somatic cells and it has been reported that mutations in a regulatory region of *abd-A*, *infraabdominal-4* (*iab-4*), which controls *abd-A* expression in A4, lack gonads (Karch et al., 1990; Cumberledge et al., 1992).

In the mutant combination of *abd-A^{M1}, an abd-A* null allele, and *iab-4^{302}*, in which A4 should be transformed into A3 (Karch et al., 1985; Cumberledge et al., 1992; Foronda, 2007) there is absence of gonads in both sexes (Fig. R. 1; Fig. R. 2); however, male genital disc derivatives evaginate normally (Fig. R. 1). Something similar occurs in females, which only showed a minor effect: oviducts of females fold towards the seminal receptacle (Fig. R. 2 B, adult).

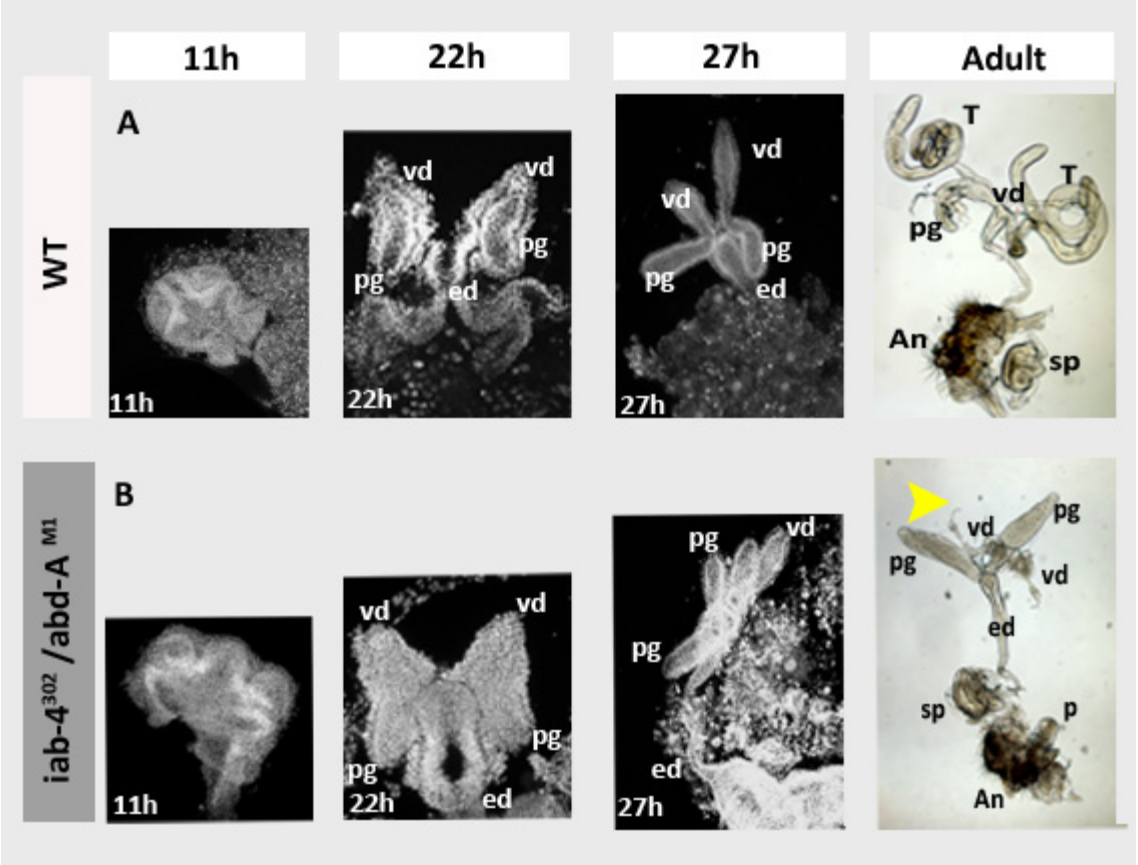


Fig. R. 1. Comparison of the development of male genital discs in wildtype and *iab-4^{302}*/ *abd-A^{M1}* mutants in pupa.

A. Wild-type development; B. iab-4302/ abd-AM1 mutant, without gonads. Anterior of the pupa to the top. Abbreviations as follows: T: Testis, vd: vas deferens, ed: ejaculatory duct, pg: paragonia, sp: spermatic pump, An: analia. Hours after puparium formation. The yellow arrowhead points to the position where testes should have been found. Note that genital derivatives develop normally in the mutant condition.

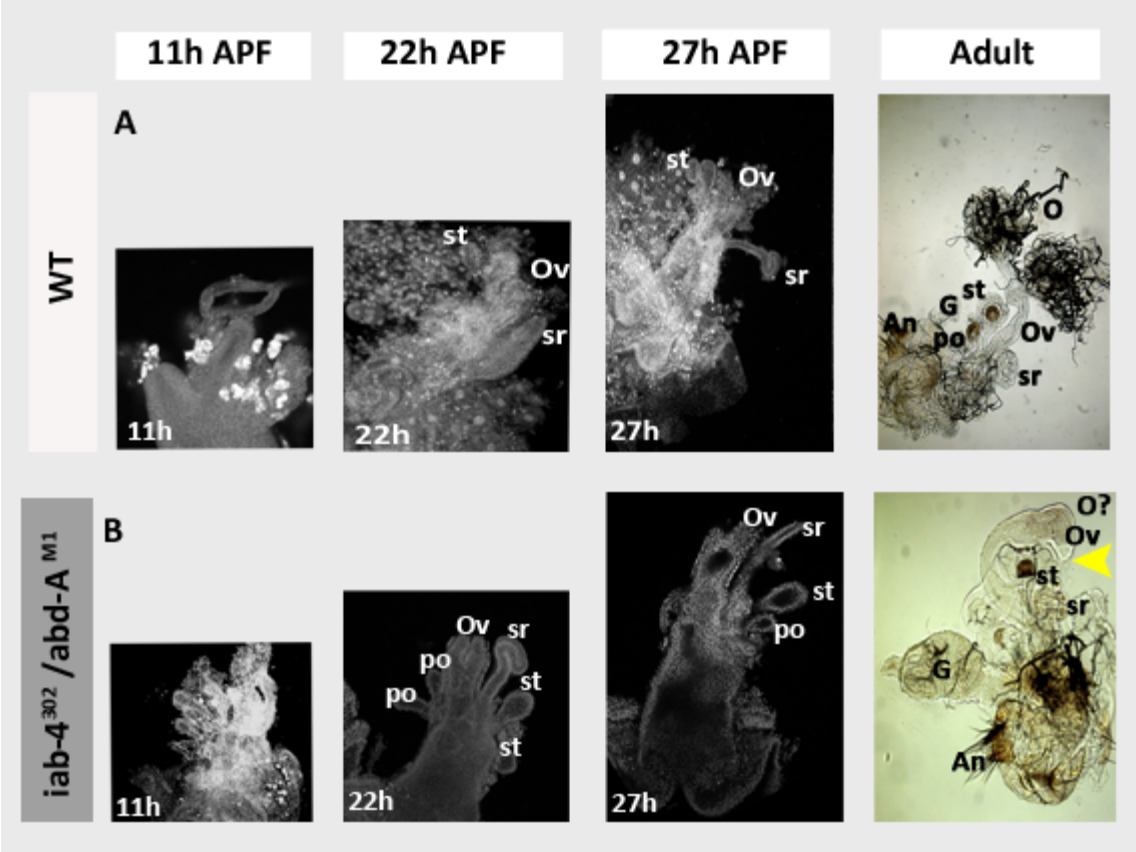


Fig. R. 2. Comparison of female genital disc development in wildtype and iab-4302/ abd-AM1 mutants in pupa.

A. Wildtype development. B. Development in the mutant. Anterior of the pupa to the top. Abbreviations as follows: O, ovary; Ov, oviduct; po, parovaria; sr, seminal receptacle; st, spermatheca; An, Analia. Hours after puparium formation. See that derivatives of the genital disc develop normally except that the oviduct turns posteriorly at the end of development (yellow arrow).

We wondered how that folding was produced, and to study it in detail we dissected females at different pupal stages. The development of the internal genitalia was normal up to 27-36h after puparium formation (APF), when the contact between oviducts and ovaries is normally produced. In the mutants, the oviducts keep on elongating towards the anterior part of the body and later their folding back begins (Fig. R. 2, yellow arrowhead in the adult), being completed at 48h APF, when the oviduct gets attached to the seminal receptacle by a filamentous structure, also observed previously (Pantelouris, 1955; Babcock, 1971a; Babcock, 1971b). We have been able to identify it as nervous tissue, since it shows expression of two different neural markers: NC82-B and HRP (Jan and Jan, 1982; Wagh et al., 2006) (Fig. R. 3 C–C", the wildtype in B-B").

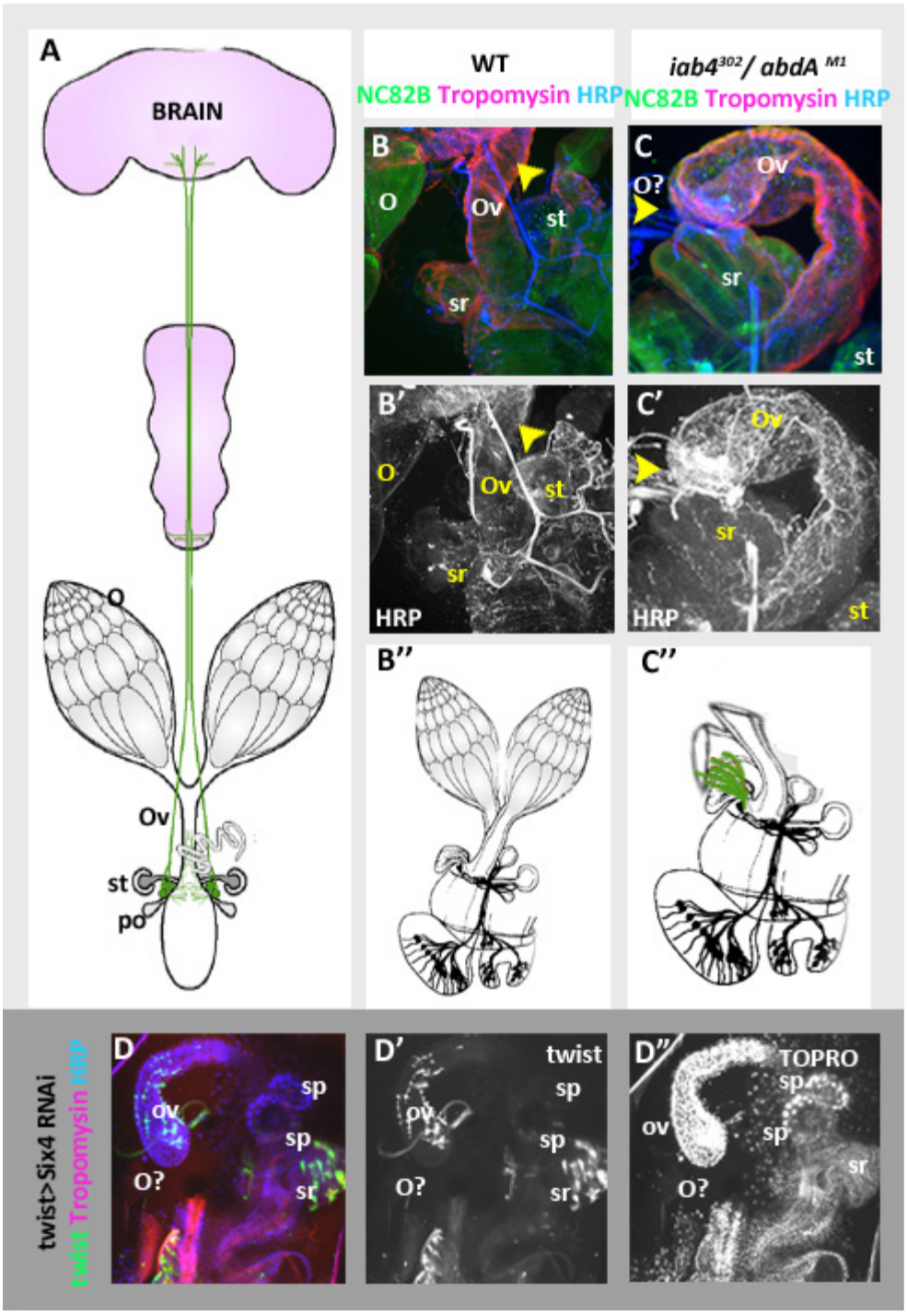


Fig. R. 3. In the absence of gonads, nerves attach the oviduct to the seminal receptacle.
A. Scheme of the innervation of the female internal genitalia. Note that there are two nerves (in green) connecting the brain to the area of attachment of spermathecae, parovaria and seminal receptacle (Häsemeyer et al., 2009). B-C". Neuromuscular staining of female adult reproductive system revealed that, while in the wildtype (B, B' and scheme B'') the nerves continue towards the ovary, in the mutant they attach the oviduct to the seminal receptacle (C, C' and scheme C'', nerves in green). Anti-NC82-B is in green, anti-HRP in blue (in white in B' and C'), both marking neural tissue, and anti-Tropomyosin, marking muscle, in red; arrowheads in (B) and (C) point to the nerves at the end of the oviduct. D-D". twist-Gal4 UAS-Six4 RNAi mutant, in which there are no ovaries and the oviducts also folded back. Twist is in green (shown in more detail in (D'), in white) and nuclei in blue (in more detail in (D''), in white). Abbreviations are as follows: O: ovary, Ov: oviduct, po: parovaria, st: spermatheca, sr: seminal receptacle. O? in (C) indicates absence of ovary.

Since *abd-A* is also expressed in the A8 of the female genital disc, in the region giving rise to the internal genitalia (Freeland and Kuhn, 1996; Casares et al., 1997; Foronda et al., 2006) it could be that the folding effect be due to a reduction of *abd-A* expression in the A8 of the mutant females. To check this, we stained these discs with an anti-Abd-A antibody and observed that the expression was similar to that of the wildtype (Fig. R. 4 A and B). It might still be possible, nevertheless, that a small reduction in Abd-A levels occurs in the mutant condition. To rule out this possibility, we induced *iab-4^{302}* clones in the female genital disc A8 segment of *y w hs-flp; FRT82B Ubi-GFP / FRT82B iab-4^{302}* larvae. As shown in the Figure R. 4 C-C", Abd-A expression was not altered in the clones. We could therefore conclude that *abd-A* expression is not modified in the disc in the mutant condition and that the effect observed is non-disc autonomous.

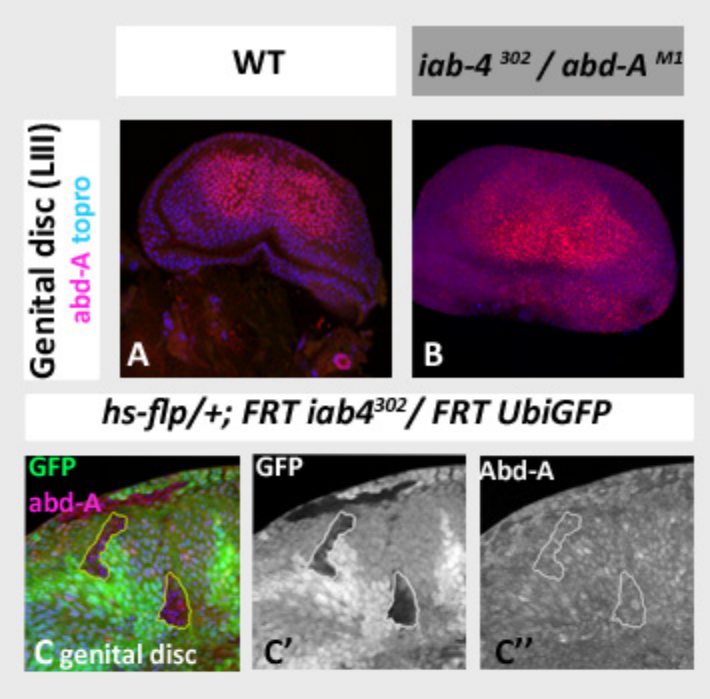


Fig. R. 4. Abd-A levels in the female genital disc are not changed in *iab-4* mutant cells.

A, B. Comparison of female genital discs of wild-type (A) and iab-4 mutants (B). Anti-Abd-A is in red, TOPRO (marking nuclei), in blue; see that Abd-A levels are not significantly reduced in the mutant. C-C''. Clones in the A8 segment of a female genital disc, marked by the loss of GFP expression (C'). The clones are delimited with a yellow or white line. Note that cells in the clones have not a different level of Abd-A expression from surrounding cells (C'').

We have observed the same folding effect in the oviducts with some other genetic combinations, such as one that inhibits the ß-integrin subunit Mys specifically in the muscle cells (Fig. R. 23), which will be discussed later, and in some other genotypes in which the gonads are absent. An example of this is found with the gene *Six4*. As mentioned before, *Six4* codes for a transcription factor expressed in gonads and muscles and required for the development of mesodermal structures (Kirby et al., 2001; Clark et al., 2006; Clark et al., 2007; Fujimoto et al., 2013). *Six4* mutants are embryonic lethal (Clark et al., 2007) but we assumed that depleting *Six4* just in the mesoderm may lead to gonads absence without lethality. In the combination *twist*-Gal4 UAS-GFP UAS-*Six4*-RNAi the *Six4*

transcription levels drop specifically in the mesoderm. In those mutants we observed absence of gonads, but only in females, a sex-specific effect which may be dependent on the *Six4* cofactor *eyes absent* (*eya*) (Neilson et al., 2010; Fujimoto et al., 2013) or on the different response of *twist* to sex (twist different response to sex was predicted in Flybase, http://.Flybase.org). In these adults the oviducts also show this folding effect (Fig. R. 3 D, D' and D'') Together with the previous result in *iab-4*^{302}/*abd-A*^{M1} mutants, we can then conclude that gonads are not required for correct genital disc evagination in males or females, but that ovaries are needed to maintain the position of the oviducts and therefore prevent the folding back phenotype seen in females.

R. 1. 1. 2 Gonad development in the absence of genital disc.

Once we studied the possible influence of gonads in the genital disc evagination, we wanted to analyze a reciprocal situation, the development of gonads in mutants lacking the genital disc. The homeotic gene *Abd-B* is expressed from abdominal segments A5 to A9 (corresponding to parasegments, 10 to 14) (Fig. R. 5 A). Within the reproductive system, *Abd-B* is expressed in the most posterior part of the gonads (DeFalco et al., 2004; DeFalco, 2005) and in the spermatocytes (Papagiannouli et al., 2014 ; Schardt et al., 2015). These authors did not find Abd-B activation in the muscles (Schardt et al., 2015); however, we have observed *Abd-B* expression in the adult muscle nuclei (Fig. R. 5 D).

Three groups of *Abd-B* transcripts were originally described: *Abd-B-m, Abd-B-r* and gamma, although the latter is now considered an *Abd-B-r* transcript (Kuziora and McGinnis, 1988; Sánchez-Herrero and Crosby, 1988; Zavortink and Sakonju, 1989). The *Abd-B-m* transcript gives rise to the Abd-B-M isoform (Celniker et al., 1990; Delorenzi and Bienz, 1990) and is localized in A5-A8 (in the genital discs, in the A8) (Fig. R. 5 B, C). Therefore, the lack of this isoform transforms A5-A8 towards A4, and hence the female genitalia (coming almost exclusively from the A8) are absent except for the parovaria, which, along with part of the uterus, derive from the A9 segment (Keisman et al., 2001; Foronda et al., 2006).

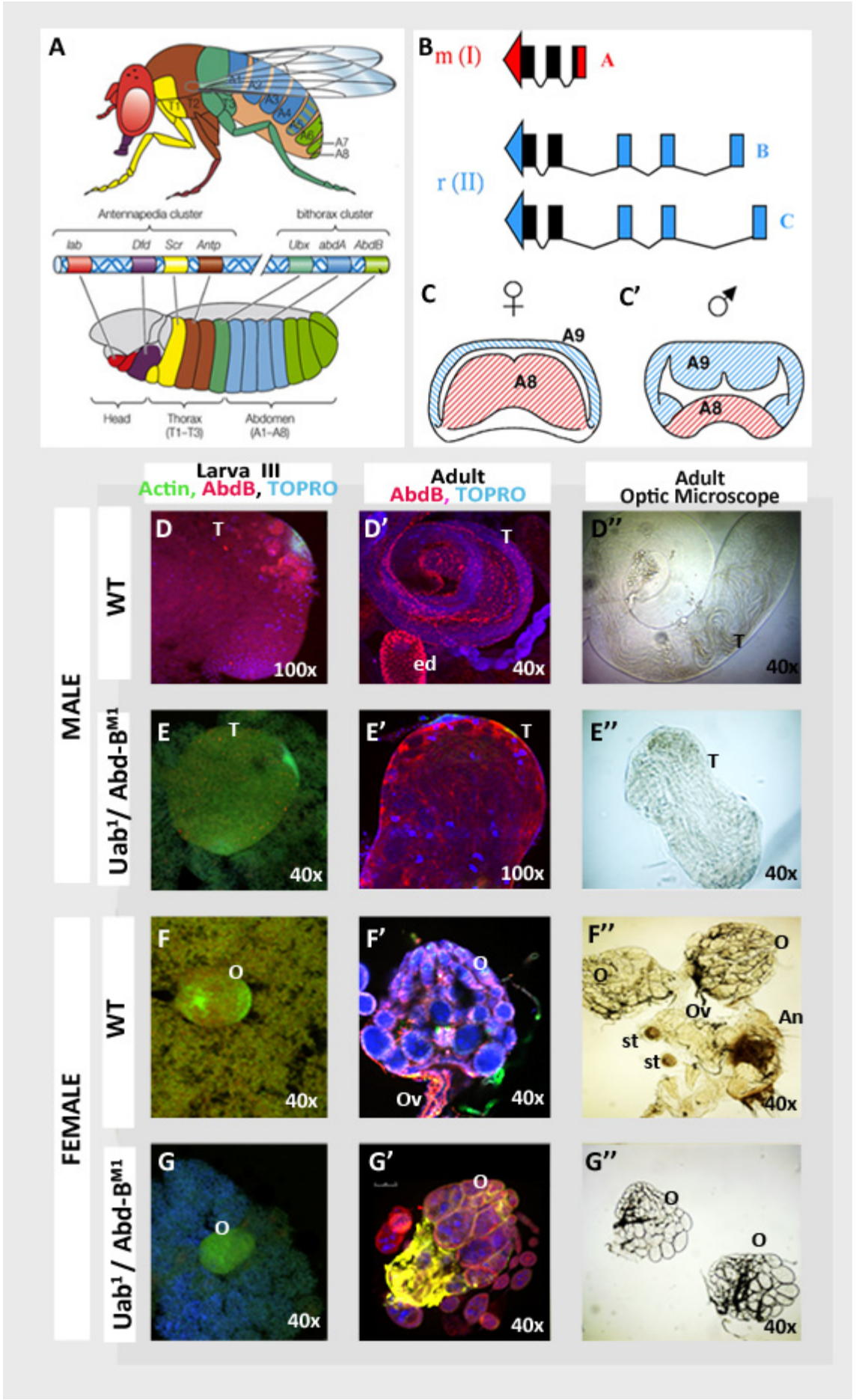
The *Abd-B-r* transcripts, giving rise to the Abd-B-R isoform, are present in the A9 segment, (Sánchez-Herrero and Crosby, 1988; Kuziora and McGinnis, 1988; Celniker et al., 1990; Delorenzi and Bienz, 1990; Casares et al., 1997; Foronda et al., 2006) (Fig. R. 5 B, C). *Abd-B-r* mutants transform in the embryo the A9 into the A8, but in adults lack genitalia both in males or females (Casanova et al., 1986). Although these mutants should not affect the female genitalia (coming from the A8), they do, and this is possibly due to the interaction

in development observed among the three primordia of the genital disc (Gorfinkiel et al., 2003), since the analia (A10) is also missing. The combination $Uab^1/Abd\text{-}B^{M1}$, a null allele for Abd-B-R (Uab^1) and a mutant defective in the activity of both isoforms ($Abd\text{-}B^{M1}$), leads to an absence of genital disc in both sexes (Casanova et al., 1986; Fig. R. 5 E- E'' and G-G'', the wildtype in D-D" and F-F"). The gonads, lacking genital disc to be connected to, are consequently unattached (Fig. R. 5 E", G", compare wit the wildtype in D" and F", respectively). We have used an anti-Abd-B antibody that recognizes both isoforms (Celniker et al., 1990) to detect if there is a significant decrease in the Abd-B protein levels in the gonads. We have observed no such decrease (Compare Fig. R. 5 D' and E'), therefore suggesting that the ovoid gonad phenotype of the $Uab^1/Abd\text{-}B^{M1}$ mutant (most evident in males, see below) is due to the lack of contact with the genital disc, and not mediated by an autonomous effect of low *Abd-B* levels in the gonad.

We have also observed in this mutant combination that female gonads, however smaller than wildtype ones, seemed correctly developed (Fig. R. 5 G-G", compare with the wildtype in F-F"), whereas male gonads remained ellipsoid, did not elongate and were not coiled (Fig. R. 5 E-E", compare with wildtype testes (T) in Fig. R. 5 D-D" and Fig. R. 1 A, adult). Both sexes showed the ability to produce gametes, as fully developed eggs and motile sperm were observed Fig. R. 5 E'' and not shown), what allow us to conclude that the gonads may be morphologically under-developed but functional. Further fertility analyses could not be carried out due to the absence of genitalia. These results, which agree with previous publications (Dobzhansky and Beadle, 1936; Stern and Hadorn, 1939), let us to conclude that testes do not develop normally in the absence of genital disc.

Fig. R. 5. Abd-B expression is not reduced in the gonads in *Abd-B* mutants that eliminate the genital disc.

A. Scheme of the expression of Hox genes in embryos (bottom) and adult (top). Abd-B is expressed at the back of the embryo. Taken from Sadava, 2008; http://www.nature.com/scitable/content/hox-genes-in-drosophila-33246. B. Abd-B transcripts and their expression in the genital disc of females (C) and males (C') drawn in a ventral view. The rectangles indicates exons and the black ones the coding regions. The Abd-B-m transcript (in red) is translated into the Abd.B-M protein, which is longer than the Abd-B-R isoform, trans. lated from the two different Abd-B-r transcripts (in blue). The Abd-B-m transcript is expressed in the A8 and Abd-B-r RNAs in the A9 of both sexes (after Foronda et al., 2006). Below, comparative development of wildtype gonads (D, D' and D", male, and F, F' and F", female) and Uab1/Abd-B mutant gonads (E, E' and E", male and G, G' and G", female) in third instar larvae and adults. Note that male gonads remain ovoid when unattached in the mutant condition (E' and E"; compare with D' and D"). Abbreviation as follows: T, testis; ed, ejaculatory duct; O, ovary; Ov, oviduct; An, analia; sp, spermatheca.


A
Antennapedia cluster
bithorax cluster
lab
Dfd
Scr
Antp
Ubx
abdA
AbdB
Head
Thorax (T1–T3)
Abdomen (A1–A8)
B
m (I)
A
r (II)
B
C
C
C'
A9
A8
Larva III
Actin, AbdB, TOPRO
Adult
AbdB, TOPRO
Adult
Optic Microscope
MALE
WT
Uab1/ Abd-BM1
FEMALE
WT
Uab1/ Abd-BM1
D
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R. 1. 2 Study of the possible role of supporting tissues in internal genitalia migration

We have not been able to answer the question of what directs the genital disc evagination towards the gonads. We wondered if there could be any sort of signal towards the disc coming from a tissue surrounding and supporting the gonads.

We therefore looked at tissues adjacent or close to gonads and genital disc, such as the posterior hindgut, and tested if it could maintain or direct migration. Previous results led us to suspect such role. It was described that the gut is directing the migration of Malpighian tubules together with the haemocytes (Ainsworth et al., 2000; Montell, 2006; Bunt et al., 2010); besides, in the male reproductive system the gut is not only the closer structure to the gonads, but it is also encircled by the ejaculatory duct as a consequence of genitalia rotation, maybe providing at the same time support and direction to this movement (Hozumi et al., 2006; Spéder et al., 2007). Since both genitalia rotation and gonad attachment to the genital disc occur almost simultaneously, we have considered that eliminating the gut, or inhibiting its growth, may affect genital disc evagination.

We then tried to eliminate or reduce the expression of genes such as *caudal* (*cad*) and *brachyntheron* (*byn*), both specific for the analia and hindgut primordia and deriving from the A10 (Kuhn et al., 1995; Wu and Lengyel, 1998; Moreno and Morata, 1999; Shinmyo et al., 2006). Fig. R. 6Fig. R. 6. A depicts the position of the genitalia segments, A8 and A9, with respect to the analia segment, A10, in a male genital disc. We tried first depleting *cad* with a UAS-*cad-RNAi* construct expressed under the control of the cad^{EM469} and cad^{MD509} Gal-4 lines, expressed in the A10 segment (Fig. R. 6 A) but found, unexpectedly, that even if the external analia was absent, the internal gut (hindgut attached to midgut) would form normally. As expected if the gut were driving genitalia migration, the development of the internal genitalia was not altered (Fig. R. 6 B) and testis were elongated and coiled (Fig. R 6 C). It may be, however, that the RNAi constructs we have used are not working as expected, since previous work with *cad* and *byn* null mutants showed both absence of external analia and malformations of the gut (Kuhn et al., 1995; Wu and Lengyel, 1998; Moreno and Morata, 1999; Shinmyo et al., 2006).

Then, we tried to prevent cell proliferation in A10 cells by expressing an *RNAi* against the *cdc2* gene, required for cell division (Prokopenko and Chia, 2005), under UAS control with the *cad*-Gal4^{EM469} and *cad*-Gal4^{MD509} drivers. The phenotype varied depending on the time of *cdc2* inhibition: depletion after larva III led to a wild-type phenotype, whereas impeding cell division early in the development (from 24h After Egg Laying (AEL) to larva III) resulted in all but one fly having no genitalia; interestingly, in such animal genitalia

migration was normal (Fig. R. 6. D) but the gonads did not lay freely inside the body cavity, but were attached to different parts of the midgut (Fig. R. 6. E, E'). The "connecting" tissue was always striated muscles that contacted the gonads but were unable to surround them (Fig. R. 6 E and, more detailed in E').

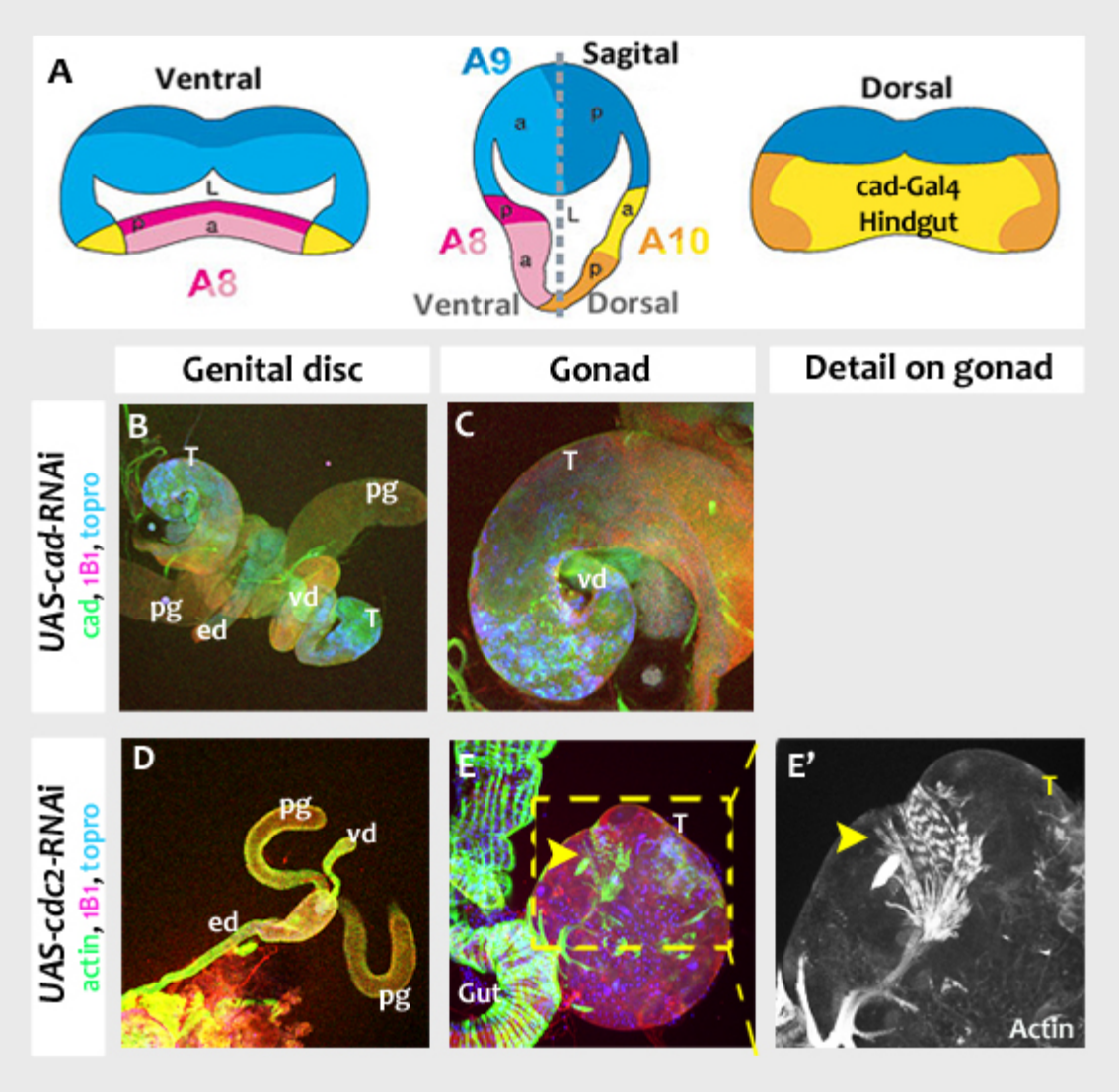


Fig. R. 6. Study of the hindgut as a supporting tissue during genital disc development.
A. Scheme of the male genital disc. Caudal is expressed in the A10, here in yellow (anterior compartment) and orange (posterior one) (after Noselli, 2006). B and C. Genital disc in which cad is reduced in the A10 (cad-Gal4 UAS-cad RNAi). Genital disc migrates normally and attach to the gonads (B), which in turn coil as in the wildtype (C). D, E and E'. cad-Gal4 UAS-cdc2 RNAi. In this combination there is inhibition of cell division in the A10 segment. Genital disc evaginates and follows the correct direction (D), but do not contact the gonads, which can attach to the hindgut (E); striated muscles from the hindgut (marked with phalloidin) migrate into the gonad, as detailed in (E'; actin in white), The yellow arrow in E and E' points to the migration of the muscles, on the surface of the ovoid gonad. In green, actin (D, E; here, in white) or cad-Gal4 UAS-GFP (B, C); in red, 1B1 (anti-Hts, marking somatic tissue of the gonad; in blue, TOPRO marks every nuclei. Abbreviations as follows: T: Testis, vd: vas deferens, ed: ejaculatory duct, pg: paragonia, sp: spermatic pump, An: analia.

Therefore, our results were inconclusive, as we could not find out why genital disc projects towards the gonads. We decided then to study the change of shape in gonads after they contact the genital disc and the need of such change, if so, for fertility. Since testes undergo major modifications in shape, we will concentrate our analysis in male gonads.

R. 2 The tissues involved in testes elongation and coiling

R. 2. 1 Elongation of the testis proceeds as genital disc muscles surround the gonad.

Previous experiments by Stern (1932) proposed that the coiling of testes depended upon their contact with genital disc, and genital disc transplants between species with different degrees of coiling demonstrated that the number of loops in the testes corresponded to that of the species that provided the genital disc (Stern and Hadorn, 1939). It was also shown that the change in morphology was a two-step process, first the testes elongate and then coil (Dobzhansky, 1930). Kozopas et al. (1992) further suggested *Wnt2* as a key regulator for modifying testes morphology, maybe through regulation of muscle determination or migration. However, no direct connection between muscle migration and coiling was reported.

We have observed that in some genotypes there was an alteration or absence of vas deferens, the structure deriving from the genital disc that contacts the testis, and that this affected testes development. Specifically, preventing cell division in the presumptive region of the vas deferens (*c855a*-Gal4 UAS-*cdc2-RNAi tub*-Gal80^{ts} males; the pattern of expression of the Gal4 line in the genital disc is shown in Fig. R. 7 B) led to different effects on testis development depending on the time of *cdc2* inhibition. When proliferation was prevented (change from 17ºC to 29ºC) just at the beginning of pupation (0h APF) the adults showed a wild-type phenotype (Fig. R. 7 A); by contrast, an earlier inhibition, from early larva III to pupa, resulted in males in which genital disc structures, including the vas deferens, were normal, but in which the testes elongated but did not fully coil (Fig. R. 7 C, D compare with Fig. R. 7 A).

Interestingly, there was a perfect correlation between the upper limit of muscle migration and the change in testis morphology, from round to elongated (the yellow arrows in Fig. R. 7 C and D mark the limit of muscle migration, as shown by strong phalloidin staining, in green). The earliest inhibition, from embryonic stages onwards, triggered a total absence of vas deferens, and therefore gonads remained rounded in shape (Fig. R. 7 E) because of

its lack of contact with the genital disc (Fig. R. 7 F; the arrow indicates where the vas deferens should have been). This implies that to attain a coiled shape, testes must first be surrounded and probably be elongated by muscle cells.

These phenotypes highlighted the importance of muscles in the change of shape of testes. We then decided to look at the nature of testis muscle, the key director of coiling.

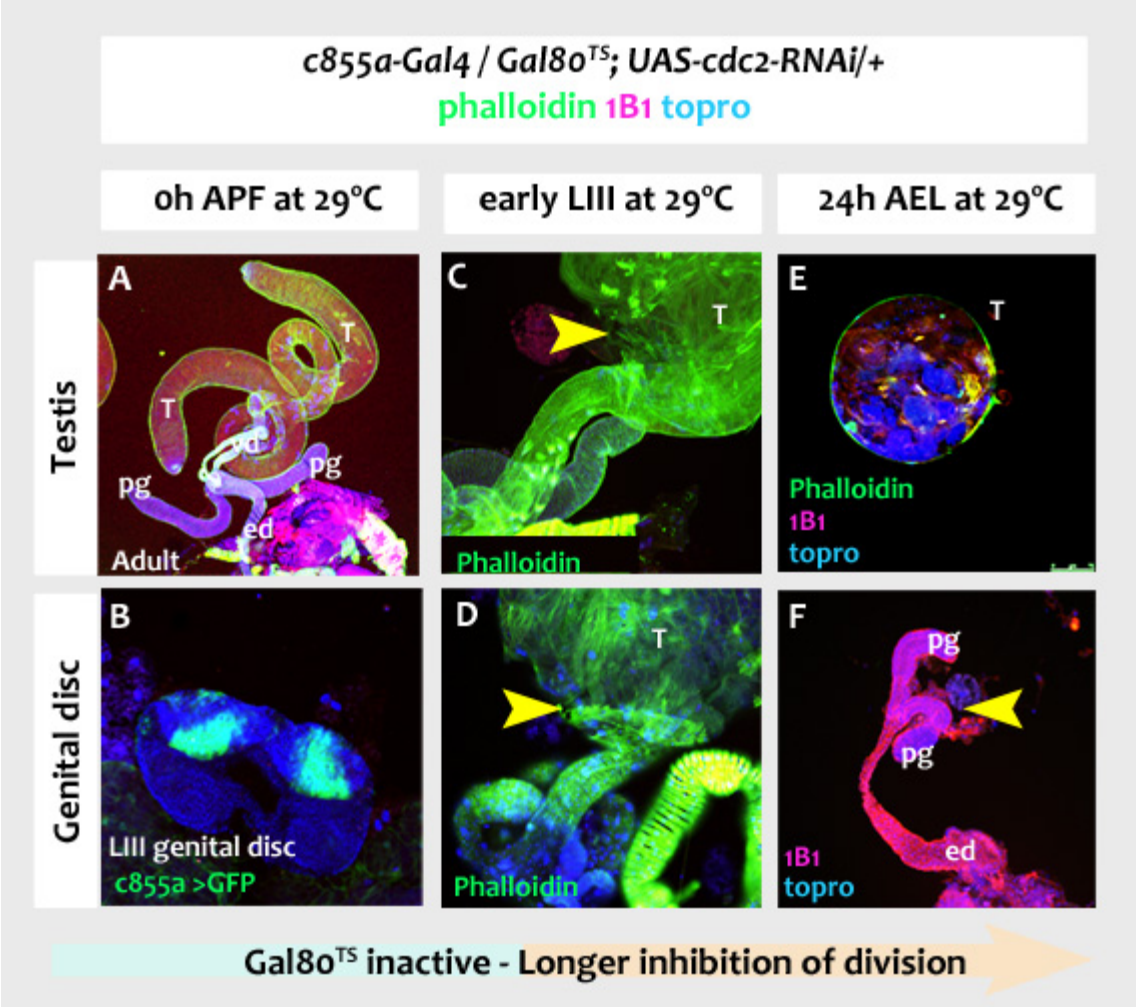


Fig. R. 7. Correlation between muscle migration and testis change of shape.
Temporally controlled inhibition of cell division with a cdc2 RNAi specifically in the vas deferens using the c855a-Gal4 line and at different times of development with the Gal4/Gal80ts system. A. Temperature shift at 0h APF. Late inhibition or no inhibition: wildtype phenotype: testes coil normally and there is no tissue defects. B shows the Gal4 pattern of the c855a-Gal4 line in the genital disc, in the vas deferens presumptive area. C and D. Early larval induction of cdc2 RNAi (by temperature shift at LIII - early pupa) and inhibition of cell division: vas deferens contacts the gonad, but testes do not coil normally. The limit of muscle migration correlates with the limit of testis change of morphology. The yellow arrows point to the limit of extension of the muscle (and of testis elongation). E and F. Induction of the RNAi at 24h AEL (development at 29ºC after the first 24h at 17ºC): early inhibition of cell division: testes remain ovoid. (E) and vas deferens does not develop (F; yellow arrow points to the place where it should have been. Abbreviation as follows: ed, ejaculatory duct; pg, paragonia; T, testis;
Phalloidin, 1B1 and TOPRO markers, as explained in the previous Figure.

These phenotypes highlighted the importance of muscles in the change of shape of testes. We then decided to look at the nature of testis muscle, the key director of coiling.

R. 2. 2 Muscles in the reproductive system and the characteristics of testis smooth muscle.

Susic-Jung et al. (2012) published, based on sarcomere organization, that there were three different types of muscle in the reproductive system: testis muscle was described as smooth multinucleated, the vas deferens muscle as striated multinucleated and the rest of the muscles as striated and mononucleated (Fig. I. 9 C). We decided to repeat their experiments and found similar results (Fig. R. 8 A-A'', B-B",). More specifically, in the testis muscle we found that actin and myosin were present and organized in parallel fibers,(and not in parallel and perpendicular fibers, as in striated muscle), and that Titin, a Z-disc specific protein, was also present in smooth muscle, although with a different pattern from that of the striated one: Titin is localized perpendicular to actin and myosin in the striated muscles, but in the testis muscle it was arranged in parallel to actin and myosin fibers; moreover, there were no perpendicular discs (Z-discs) or any other protein that accumulated perpendicularly to the actin and myosin fibers (Figure I. 9 B and Fig. R. 8 A and B). Therefore, it can be concluded that testis muscle fibers are a very special type of muscle that may be considered as smooth because it does not have a clear Z disc, or an altered striated one, as the Titin fibers are parallel to the rest of the fibers instead of perpendicular to them (Bairati, 1967).

R. 2. 2. 1 Genetic definition of smooth muscle

The muscle identity genes are specific for each tissue; thus, the striated jump muscle of the leg needs a set of genes different from that of the indirect flight muscle (Baylies et al, 1998; Maqbool and Jagla, 2007). Since smooth muscle is probably the driving force in changing the morphology of the testis and its description was only morphological (Susic-Jung et al., 2012), we decided to study it in more detail. The muscle surrounding the testes derives from the vas deferens, which, in turn, evaginates from the genital disc mesodermal cups. The definition of this muscle as visceral, somatic or even cardiac has not been addressed, nor has its genetic identity requirements. To check which selector genes were expressed and required for these muscles we have used some Gal4 lines specific of muscles, or more specifically, of myoblasts (1151-Gal4) (Soler et al., 2012) (Fig. R. 8 C -C"), or expressed in epithelium and muscle of the vas deferens and testis muscle (*c855a*-Gal4) (Sözen et al.,

1997) (Fig. R. 7 B, Fig. R. 8 D, D', E-E"), and RNAi constructs (under UAS control) specific for some genes needed in muscle development. We have assessed the requirement of each gene by analyzing the organization of microfilaments into a sarcomere, and comparing it to a normal striated muscle sarcomere; we have also observed the extent of testes coiling.

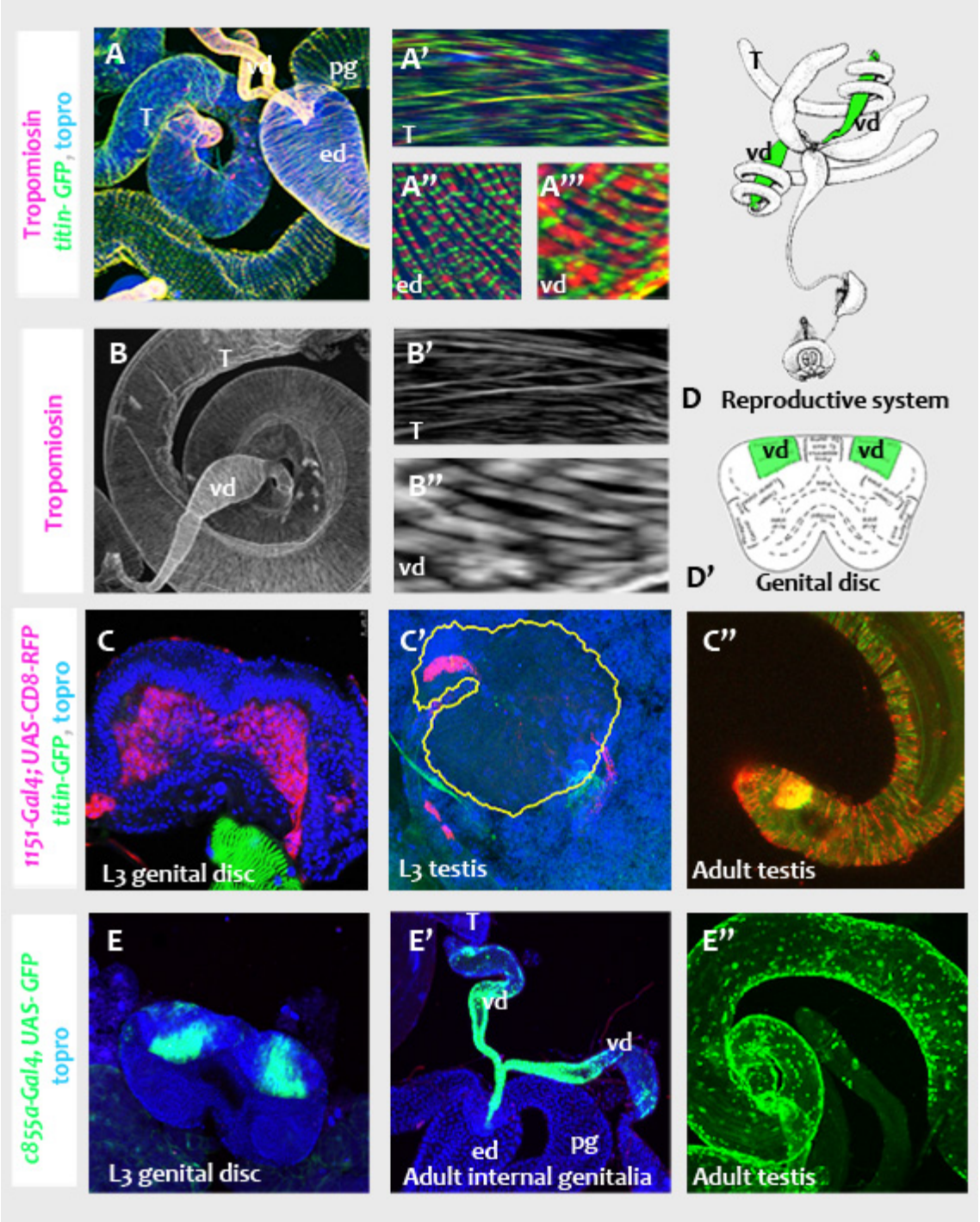


Fig. R. 8. Comparison of some different Gal4 lines selected in this thesis, the antibodies used to detect the muscle type and description of muscle types in the male reproductive system.

A-A'''. Structures of the male reproductive system with different types of muscles. ,Note the different disposition of tropomyosin (in red) and Titin (in green) in Testis (T) (A') as compared to

ejaculatory duct (ed) (A'') and vas deferens (vd) (A'''). B Tropomyosin disposition in testis and vas deferens. See it in more detail in B' and B'', testis on top (B'), vas deferens at the bottom (B''). C-C", 1151-Gal4 UAS-CD8RFP male genital disc and testis. RFP expression in the genital disc (C), and larval (C', larval testis surrounded by a yellow line) and adult (C'') testis; differentiated muscles are recognized by the presence of Titin (in green) while myoblast are marked in red. Note that muscles begin their differentiation and start expressing Titin, in green, which explains the lower Titin expression, marking undifferentiated muscles. D, D'. Scheme showing in green the expression driven by the c855a-Gal4 line in the adult (D) and the region of the genital disc that originates it (D'). E Expression of GFP in the genital disc driven by the c855a-Gal4 line, showing the prospective area of vas deferens. E' Adult internal male genitalia of a c855a-Gal4 UAS-GFP fly showing GFP expression in the vas deferens. E'' Muscle nuclei of a c855a-Gal4 UAS-GFP fly are visible in the adult testis. Abbreviation as follows: T: Testis, vd: vas deferens, ed: ejaculatory duct, pg: paragonia, sp: spermatic pump.

Inhibition of visceral muscle selector genes such as *Anaplasic lymphoma kinase* (*Alk*), the *jelly belly* receptor (*jeb*) (Lee et al., 2006), or *biniou* (*bin*) (Taylor, 2000), only triggered minor fusion defects (Fig. 9 A, A' and not shown). *Myocyte enhancer factor-2* (*Mef2)*, known to be required for adult muscle differentiation (Taylor, 2006; Sandmann et al., 2007; Susic-Jung et al., 2012; Soler et al., 2012), seemed not to be needed in the reproductive system, either, as its depletion with the vas deferens *c855a*-Gal4 or myoblasts 1151-Gal4 lines resulted in normal reproductive system structures and muscles (Fig. R. 9 B, B' and not shown); these mutant combinations, nevertheless, produced strong phenotypes in locomotive system, and the pharates were unable to emerge from the pupal case. The tandem of homeobox-containing genes *ladybird early* (*lbe*) and *ladybird late* (*lbl*), participates in determining somatic muscles (Jagla et al., 1998b; Junion et al., 2007); however, *lbl* was not actively transcribed in the vas deferens, as shown by lack of expression of a *lbl* –lacZ reporter (De Graeve et al., 2012) and we have found that the lbe -lacZ element does not show any signal either (Fig. R. 9 C and C').

Some other selector genes have been identified in different muscle types: for example, *Drop* (*Dr*), also known as muscular homeobox gene (*msh*) (Nose et al., , 1998; Paululat et al. 1999; Dobi et al., 2011) plays a role in the determination of pigment cells of the eye (Tearle et al., 1994) and of both oblique and transversal abdominal muscles of segments A1 to A7 (Nose et al., 1998), and *Six4* is required in the ventral longitudinal and the oblique muscles in the embryonic abdomen and in the adult leg muscles (Jagla et al., 1998; Jagla et al., 1999; Clark et al., 2006b; Maqbool and Jagla, 2007). We looked at the expression and requirement of these two genes because they had been reported to be active at some stage of the development in the reproductive system (Kirby et al. 2001; Ahmad & Baker 2002; Clark et al. 2007; Chatterjee et al. 2011)

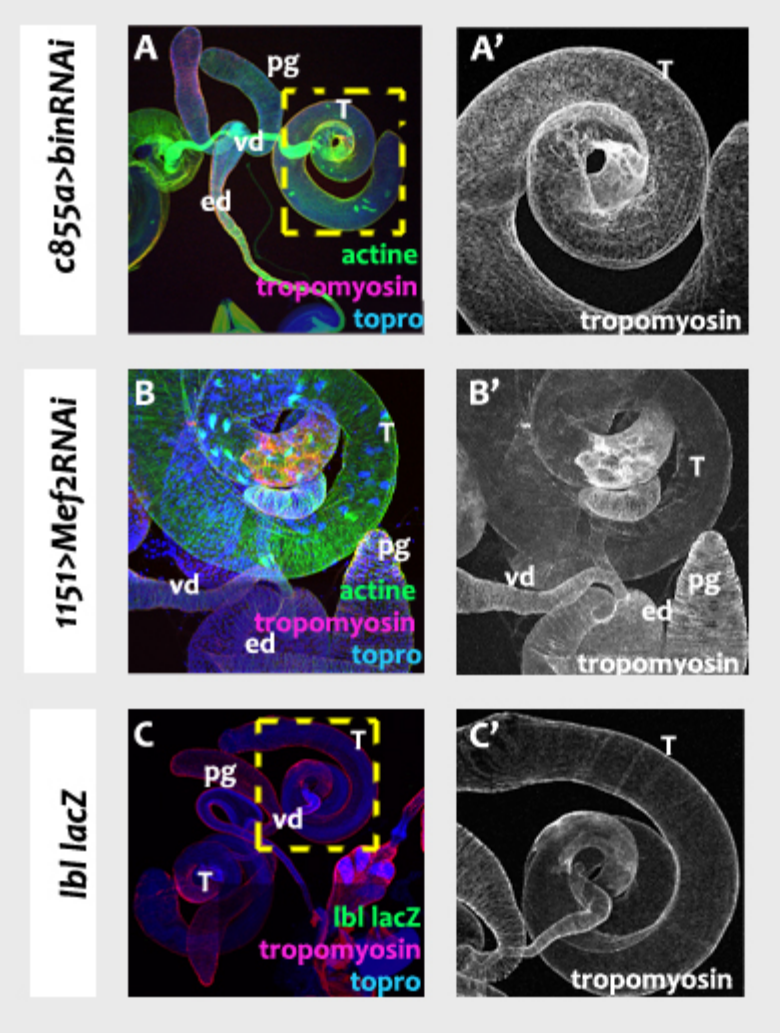


Fig. R. 9. Some muscle selector genes are not expressed in the testis muscle or do not present the expected phenotype. A. Inhibition of bin in the vas deferens (c855a-Gal4 UAS-bin RNAi adults) did not trigger any muscle defects, as seen with actin (in green) and tropomyosin (in red). TOPRO is in blue. A'. Testis close-up. B, B'. Inhibition of Mef2 in myoblasts (1151-Gal4 UAS-Mef2 RNAi animals) had no muscular defects (staining as in A, A'),. C. lbl is not expressed in the muscle cells (lbl-lacZ, in green) and there are not muscular defects, as shown by staining with tropomyosin, in red in C, in white in C' (a close-up is shown in this panel). Abbreviations a sin previous Figures.

Dr activates the transcription of *branchless* (*bnl*), which is the signal required for the migration of mesodermal cells into the mesodermal cups of the male genital disc (Ahmad and Baker, 2002; Chatterjee et al., 2011). As explained before, the mesodermal cups will give rise to the vas deferens, and therefore the muscles of the testis (Fig. R. 8 D and D'). When we inhibited *Dr* by using the vas deferens Gal4-line (*c855a*-Gal4) we observed aberrant muscles encircling the testes, and these were unable to elongate and coil normally (Fig. R. 10. C, C', ,compare with the wildtype in Fig. R. 10 A and B).

Six4 is a gene with many functions, as it collaborates in the development of the ventral mesoderm (Clark et al., 2006), eye (Seimiya and Gehring, 2000; Anderson et al, 2012), muscle (Clark et al., 2006; Jemc and Rebay, 2007) and gonads (Kirby et al., 2001; Clark et al., 2007), with similar functions in the gonads to those of its human homologue, DUX4 (Leung and Wagner, 2013). Depleting *Six4* in the prospective area of the vas deferens, the mesodermal cups, with the *c855a*-Gal4 line, led to two different testis phenotypes

depending on whether or not there was contact of vas deferens and testis. An incomplete coiling was observed when the vas deferens had contacted the gonad (Fig. R. 10. D and D') and in this case the muscles of the testis show a striated morphology. However, when there was no contact, the unattached gonads associate with the hindgut from which striated muscle fibers could migrate into the testes, similar to what happened when there was no genitalia in the hindgut experiments (Not shown and Fig. R. 6).

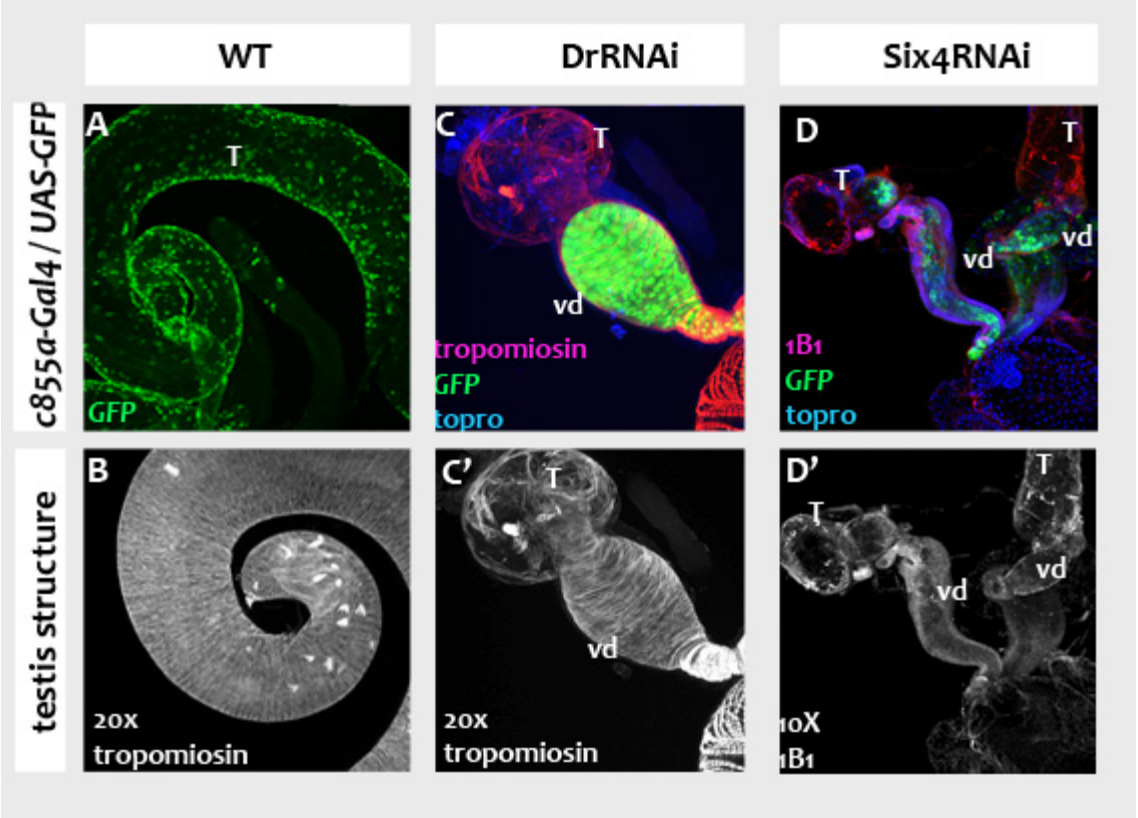


Fig. R. 10. Smooth testis muscle needs *Dr* and *Six* 4 for its correct definition.

A. c855a-Gal4 line expression in the muscle nuclei of the adult testis. Note normal coiled shape of the testis and normal pattern of tropomyosin in the testis muscle (B). C. Inhibition of the homeobox-containing gene Dr by its RNAi in the muscle cells of the testis. Note that testis (T) is rounded in shape and observe also the disorganized muscle pattern, as marked by tropomyosin, in red, and shown in a single channel in C'. D. Inhibition of Six4 gene specifically in the muscle cells of the testis had similar results as Dr depletion; note that testis do not reach normal coiled shape; more clearly shown in D'. In this case, we have used anti-Hts (1B1 antibody) (Ding et al., 1993) to mark gonadal tissues (Yue and Spradling, 1992). Abbreviations as follows: T: Testis, vd: vas deferens.

As we obtained similar results depleting *Six4* and *Dr* genes with the *c855a*-Gal4 and 1*151*-Gal4 lines (Fig. R. 10 and not shown) we can propose that these two genes are required for the correct development of the testis muscle and, subsequently, testis morphology. Although we conclude that *Six4* and *Dr* are necessary for smooth muscle development, due to lack of proper tools to carry out gain-of-function experiments we cannot determine if the expression of these genes is sufficient to form smooth muscle.

Abd-B is expressed in gonadal structures of the fruitfly (Nanda et al., 2009; Papagiannouli et al., 2014; Papagiannouli and Lohmann, 2015), and we have seen its expression in testis muscles, so we decided to check its requirement in the development of genital muscles. When we inactivated the gene with and *Abd-B*-RNAi line directed by the 1151-Gal4 driver, every genital disc derivative was normal except the vasa deferentia, which were absent, and in this case testes were attached to the ejaculatory duct (Fig. R. 11. A). The shape of the testis remained rounded and no coiling was observed. In these cases, testes were covered by muscles in which we recognized parallel and perpendicular fibers reminding the sarcomere organization of striated muscle instead of the wildtype smooth muscle of the testis (Fig. R. 11 B and B' and more detailed in D; compare with the wildtype in C).

Finally, to test previous hypothesis by Kozopas et al. (1998), who proposed *Wnt2* as an important gene for testes development, we inhibited this gene on the vas deferens prospective area (with the *c855a*-Gal4 line); fresh dissection showed coiled but less or not pigmented testes. We only found a case (out of eight) where the inhibition of *Wnt2* in the genital disc with a Gal4-line driving broad expression in the genital disc (*c355*-Gal4) resulted in one testis failing to coil.

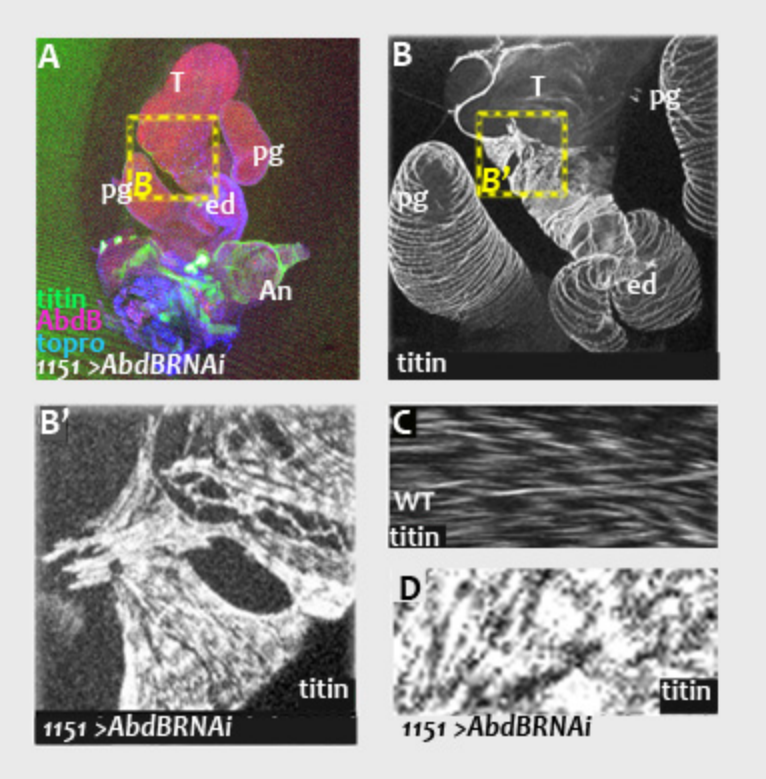
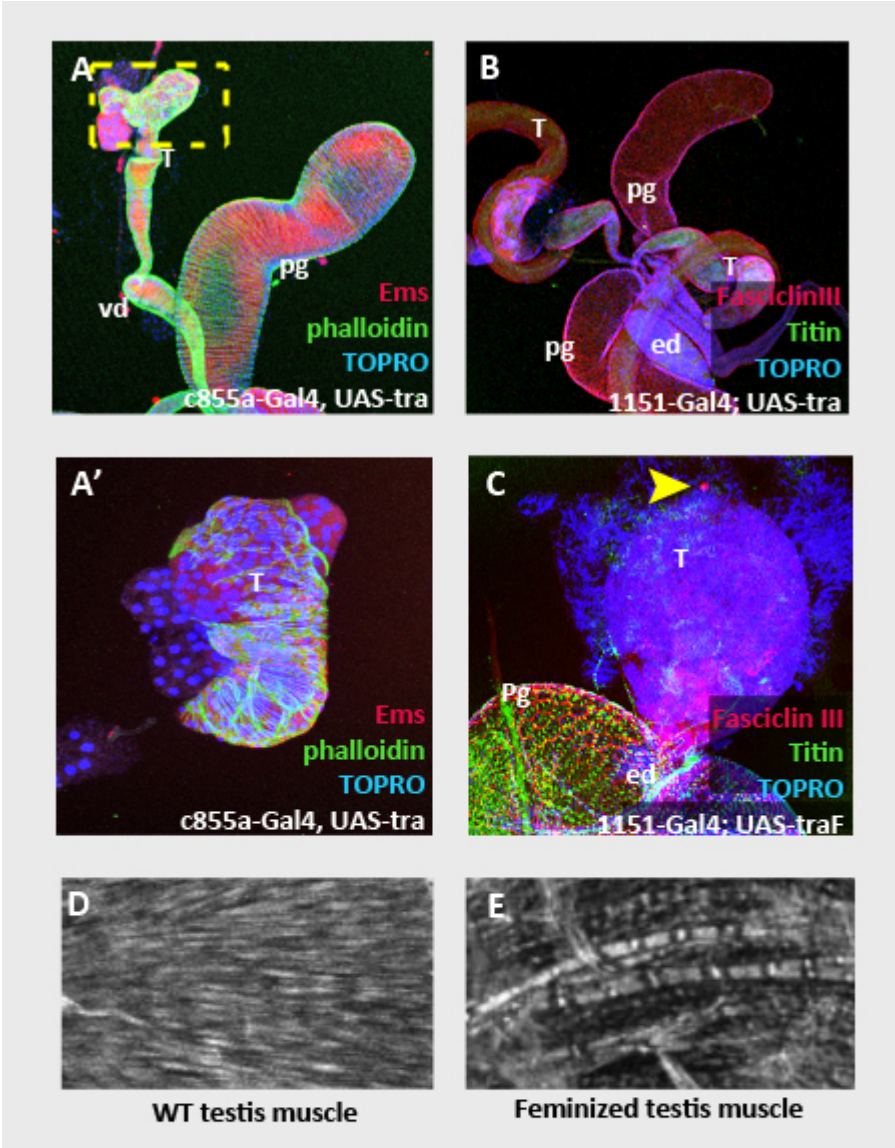


Fig. R. 11. *Abd-B* is required for the correct development of the muscles of the testis.
A. Inhibition of Abd-B in the myoblasts with the 1151-Gal4 line. Note that testes (T) are not coiled and vas deferens is absent, but the rest of the internal genitalia are normal. B. Detail of the mutant testis. B' Further detail of muscle of the testis, a sarcomere pattern characteristic of striated muscle, may be observed. C, D. Muscle of the wild-type testis (C) as compared to muscle of mutant genotype (D). Note that Titin-GFP shows a sarcomere organization characterictic of smooth muscle in D. Titin is in green in A and in white in B–D; AbdB is in red in A, TOPRO is in blue in A. Abbreviation as follows: T: Testis, ed: ejaculatory duct, pg: paragonia, An: Analia.

As the unique smooth muscle in Drosophila is observed in the reproductive system of males, we wanted to know if a change of sex might alter muscle development. Therefore, we expressed in males the gene *tra*, a sex-determining gene only active in females, using the myoblast *Gal4* line (*1151*-Gal4) or the vas deferens c855a-Gal4 line to change the sex of the muscles: we observed testes that do not fully elongate and coil (Fig. R. 12 A, A' and B, B') and also, again, changes in the disposition of the sarcomere filaments that could be interpreted as changes towards a striated muscle organization (Fig. R. 12 C and C', compare to Fig. R. 10 C' and D' and Fig. I. 9).

Fig. R. 12. Testis muscles require correct sex determination.

A-D. Transformation of vas deferens (vd) muscles from male to female in c855a-Gal4 UAS-tra (A) or 1151-Gal4 UAS-tra (B) pupae results in uncoiled testis (T). Depicted in more detailed in A' and C. Muscles of these feminized testes were marked with phalloidin in A and A' or Titin in B and C, and the filaments do not show a disposition similar to wildtype (D, phalloidin) but rather show a sarcomere organization similar to striated muscles (E, phalloidin). Muscles are marked in green (phalloidin (in A and A') and titin (in B and C)), PCs are marked in red (Ems) in A and A', hub in red (Fasciclin III, in B and C), nuclei in blue (TOPRO) in A-C yellow arrow in C points to the hub (fasciclin III in red)



As a result of using a variety of RNAi constructs and two different Gal4 lines we have found that the smooth muscles that surround the testes need a correct expression of *Six4, Dr* and *Abd-B*, and the activity of the male sex-specific pathway; if the expression of these genes is not the wildtype one, the muscle is transformed into what seems striated muscle, and in the mutant combinations the testes do not elongate and coil as in the wildtype, suggesting that smooth muscle identity may be required for these morphogenetic events.

R. 2. 3 Notch signaling pathway is active in the smooth muscle.

The development of the internal genitalia muscles and of testes suggests there should be communication between different tissues. Previous results indicated a role of different signaling pathways in muscle development. Thus, Notch has been reported to be active in some muscles and needed for muscle migration during metamorphosis (Dutta and VijayRaghavan, 2006); Notch is also required for lateral inhibition and, like every signaling pathway, it is involved in cell fate and patterning (Anant et al., 1998; Housden et al., 2014; Bray, 2014). The EGFR pathway is also required in patterning and morphogenesis (Perrimon et al., 2012), known to be active in muscles, and also related, in vertebrates to the contraction by a smooth muscle of a fetal conduct (Hong et al., 2014); in the Drosophila female genital muscles, contraction and development of muscles depends on FGF signaling and is related to oogenesis and fertility (Irizarry and Stathopoulos, 2015). The JAK/STAT pathway direct target *tinman* is a well-known muscle selector gene that helps in the activation of muscle identity genes, such as *slouch* or *apterous* in the ventral muscles, *Six4,* defining abdominal dorso-ventral muscles, and *eya*, needed in the dorsal, ventral and lateral muscles in the abdomen (Liu et al., 2009). Moreover, the JAK/STAT and JNK pathways could be involved in muscular atrophy (Kucherenko et al., 2011; Piccirillo et al., 2014). Finally, Kozopas et al. (1998) reported that *Wnt2* was transcribed in the vas deferens edge, where muscles would divide and from where they would migrate to contact the gonad. The Wnt pathway is involved in several cellular mechanisms like migration, proliferation or Planar Cell Polarity, and therefore, as with the other pathways, it may be required for testes smooth muscle development (Fig. I. 9).

We have used several readouts to ascertain the possible role of these pathways and found a clear activity of the Notch one. Notch is active in a small population of cells in the larval testis (Fig. R. 13 A and A') and in the leading edge of the evaginating genital disc at 6h APF (Fig. R. 13 B and B'). In the adult, *Enhancer of split E*(*spl*),,a target of the Notch pathway, is active not only in the vas deferens and the testis (Fig. R. 13 C), but also in in stem and cyst cells of the gonad and in the binucleated secretory cells of the paragonia (Fig. R. 13 C and

C'). The JNK and JAK/STAT pathways were not active in the muscles but in the pigment cells and this will be described in the next section

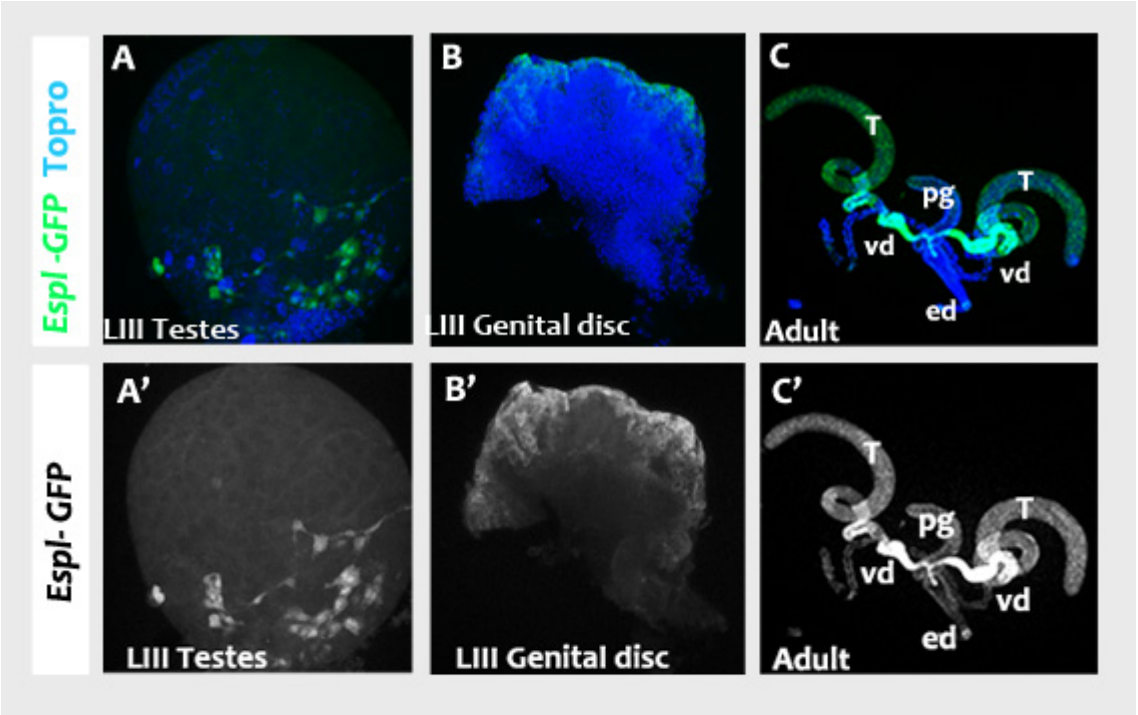


Fig. R. 13. *E*(*spl*) expression, as a Notch signaling pathway read-out in the genital disc and testes.

A, A'. E(spl) is transcribed by a small population of cells in the larval testes and in the genital disc (B, B'). E(spl) expressing cells are localized mainly in the leading edge of the disc, before it evaginates. In the adult (C, C'), E(spl) is transcribed in the vas deferens (vd, bright green) and testis (T). There is also a small population of cells inside the ejaculatory duct, where both vas deferentia originate (Abbreviation as follows: T: Testis, vd: vas deferens, ed: ejaculatory duct, pg: paragonia.

After demonstrating Notch activity in the testis muscle, we wanted to know if the inhibition of this pathway might trigger a change in its pattern. This was the case. We have inhibited the Notch pathway specifically in the vas deferens (with the *c855a*-Gal4 line) by expressing *Beaded of Goldschmidt* (BD^{G}), a truncated form of an allele of the Notch ligand *Serrate* (Hukrikede et al., 1997; Mummery-Widmer et al., 2009) (Fig. R. 14 A, A' and A''), a truncated Dominant Negative form of *Mastermind* (Mam^{DN}) (Helms et al., 1999) (Fig. R. 14 B, B' and B'') or the Notch extracellular domain of this protein (*Necd*) (de Celis et al., 1993) (not shown), all of them proved to inactivate the Notch pathway (Zacharioudaki and Bray, 2014). In all the cases we find morphological defects at the tip of the testis, which may relate to muscles failing to attach correctly.

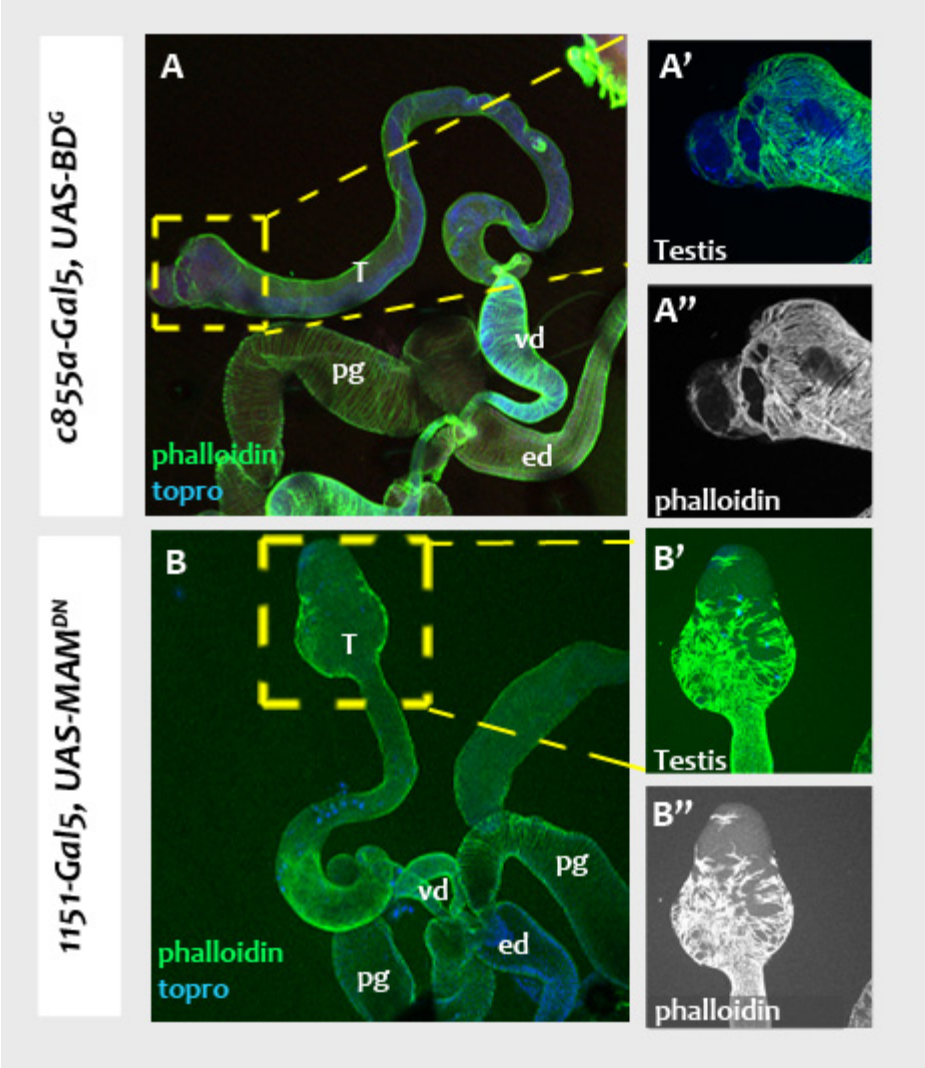


Fig. R. 14. Notch inhibition in muscles of the reproductive system.
A-A''. Inhibition of Notch in the prospective area of the vas deferens leads to an aberrant morphology of the testes (A, shown in more detail in A' and A''). B-B'' Reducing Notch activity in every muscle resulted again in testis malformation (B, a close-up of the marked region is shown in B' and B''). Note that in every case muscles, seen with phalloidin in green (A' and B') and white (A'' and B''), were unable to fully encircle the testis. Abreviations and staining, as described in previous Figures.

In the course of our experiments we have become aware of a region located in the insertion of the vas defers to the ejaculatory duct that may be hosting muscular stem-like cells, because they maintain twist expression and Notch activation (Piccirillo et al., 2014, Fig. R. 14). If so, this could be the first description of muscle stem-like cells in the reproductive system of males. We wondered if this region should have a specific niche and therefore, a specific microenvironment.

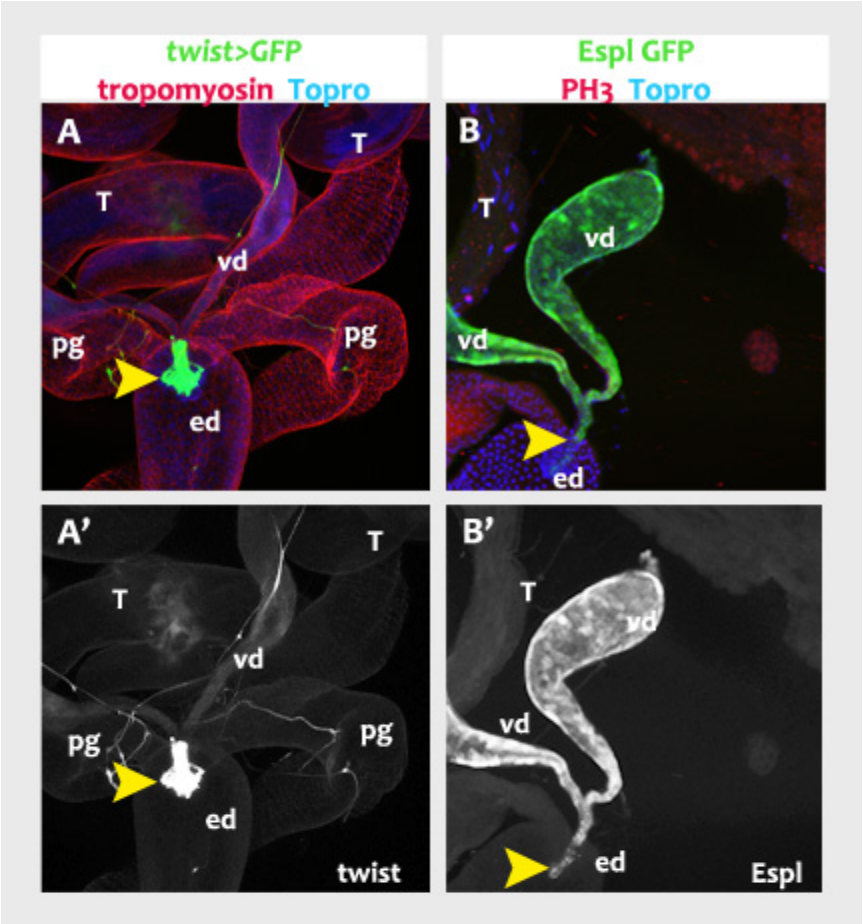


Fig. R. 15. Probable muscle stem-like cells found at the vas deferens insertion point in the ejaculatory duct.

A, B. Sustained expression of twist-Gal4 UAS-GFP (A and A') and expression of E(spl) detected with a *E(spl)mβ-CD*2 reporter (Celis et al. 1998) (B and B') at the point of insertion of the vas deferens in the ejaculatory duct, indicated with yellow arrows. In A we show tropomyosin expression and in B, PH3 to detect proliferation due to Notch signalling TOPRO in both. Abbreviation as follows: T: Testis, vd: vas deferens, ed: ejaculatory duct, pg: paragonia.

We have demonstrated, so far, a requirement of several genes (*Dr*, *Six4*, *Abd-B*) and two pathways (the sex determination and Notch pathways) in testis muscle definition and testis development. The environment, as explained in the Introduction, is also important for the specification of the muscle. Therefore, we wanted to study the tissues that may have an input in muscle determination; we will concentrate in the nervous system and Pigment Cells (PCs), as these are the closest tissues to the testis muscle.

R. 2. 4 Innervation of the testis muscles.

The morphological and genetic description provides, however, no information about the physiological or functional properties of the smooth muscle of *Drosophila.* As testis muscle is the only smooth muscle described in the fruitfly, we have no possible comparison with

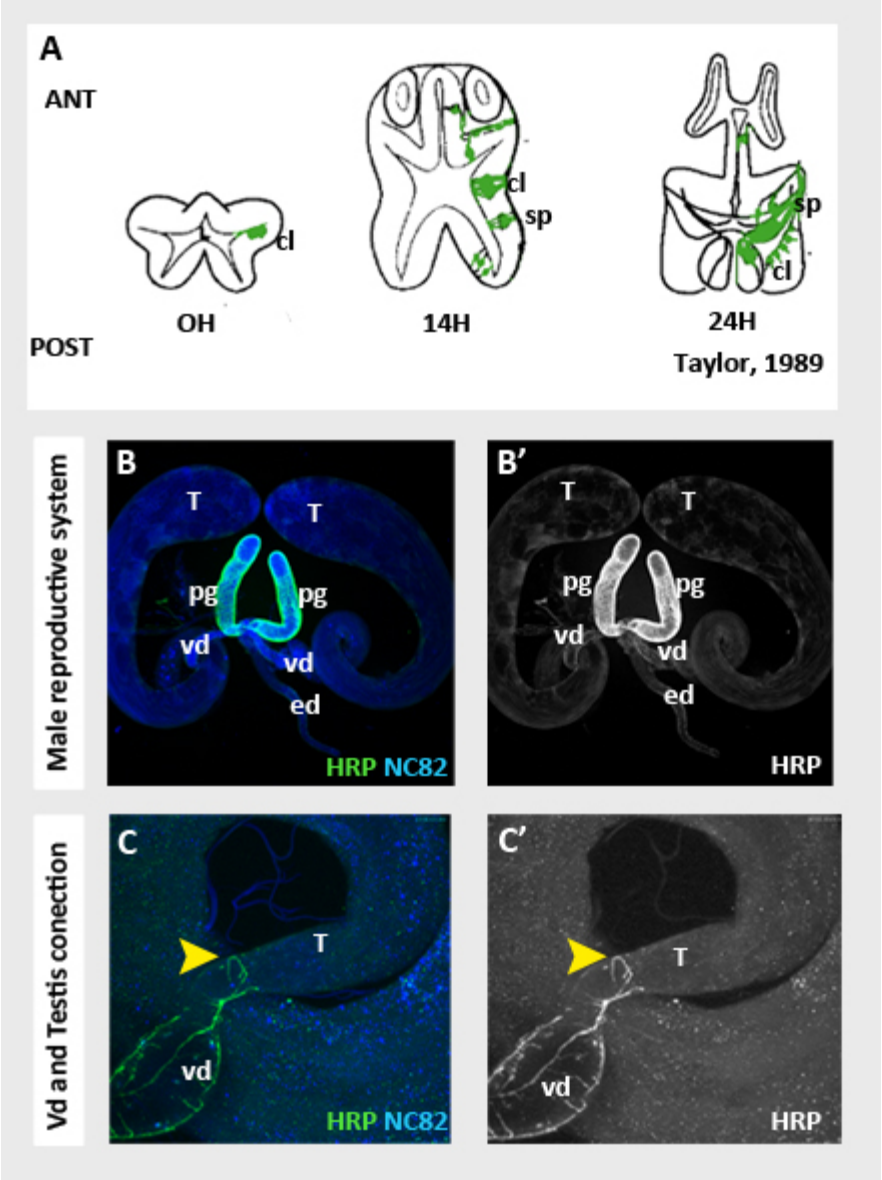
other muscles in this species, but we can look to the properties of smooth muscles in vertebrates. In general, vertebrate smooth muscles are thought to have a slower though more sustained reflex contraction. By contrast, striated muscles are finely controlled by the nervous system, both in vertebrates and in insects, so that they respond quickly to an electrical input to contract, e.g. the indirect flight muscle (Taylor, 2000).

Taylor (1989) studied the innervation of the reproductive system during pupation, and she observed that the innervation of the male genital disc starts at the origin of the clasper(cl) (Fig. R. 16 A, 0h, in green), that at 14h the nerves colonized the spermatic pump (sp) (Fig. R. 16 A, 14h, in green) and that the union to apodeme occurs at 24h (Fig. R. 16 A 24h, in green). However, she did not find innervation in the testis. Thus, we tried to verify her results with a staining of the reproductive system with HRP (Jan and Jan, 1982), NC32 (Wagh et al., 2006), or tropomyosin, markers of neurons (the two former) and muscles (the latter). The staining showed no nerves in the testis except at their base, where they contact the vas deferens (Fig. R. 16 B and B', more detailed in C and C'). The results agree, therefore, with the description of smooth muscles as not needing innervation or responding to a voluntary contraction.

The role of the nervous system in our model could be then the guidance of muscles. The central nervous system in Drosophila adults is located ventrally in the thorax, except the brain, and from there many nerves migrate to contact every region that needs innervation. Therefore, muscles coming from the genital disc might follow the path described by nerves coming from the thorax during metamorphosis (Dutta and VijayRaghavan, 2006).

In summary, we have shown that nerves innervate the paragonia and the vasa deferentia but not the testis. Therefore, nerves may be required for striated muscle identity, and the absence of nerves in muscles surrounding the testes agrees with their being smooth muscle.

Fig. R. 16. Innervation of male reproductive system.
A. Scheme of the innervation of the male internal genitalia. Note that the nerves (in green) are only depicted in half of the genital disc at the different time points. There is one innervation point at the insertion of vas deferens (vd) in the ejaculatory duct (modified from (Taylor 1989). B neuromuscular staining of male adult reproductive system revealed that paragonia (pg) are strongly innervated (B and B') while there is little innervation at the vas deferens (vd) and it is discontinued at the attachment with testis (C and C') Anti-NC82-B, is in blue, anti-HRP in green (in white in B' and C'), both marking neural tissue. Abbreviation as follows: T: Testis, vd: vas deferens, ed: ejaculatory duct, pg: paragonia



R. 2. 5 Pigment Cells role in smooth muscle cell specification and testis development.

Pigment cells (PCs) are a group of cells deriving from the fat body that, together with smooth muscles, assemble the testis sheath. Previous experiments have described an effect of PCs alteration on testis development (Kozopas et al., 1998; DeFalco et al., 2008) ,probably done through the intervening muscle layer (see Fig. I. 4). Therefore, we decided to study muscle morphology when PCs development is compromised.

R. 2. 5. 1 Attempts to eliminate Pigment Cells to determine their function.

To understand if PCs were determinant for smooth muscle fate, we decided to try and eliminate PCs. If these were required for muscle development, their absence would trigger loss of smooth muscle fate and changes in tests development. We have first analyzed previously published mutant combinations lacking PCs.

Wnt2 mutants have less PCs than the wild-type and their genital discs fail to contact the gonads (Fig. R. 17 A, B and B'; Kozopas et al. 1998). Nanda et al. (2009) described that *SOX100B* mutants also lacked PCs and were not able to develop coiled testes (Fig. R. 17 D, E, E'). We tried to reproduce their results and looked carefully at the sarcomere organization of the muscle layer structure. However, we have found normal coiled testes in the same published combinations of either *Wnt2* (*Dwnt2*^{RJ} and *DWnt2 ems1(Df(2R)11)* (Fig. R. 17 C) or *SOX100B* mutants: (*j3B9*(*rv12*)), *P(dco+)tlle-2)* (Fig. R. 17 F). Fresh dissections of the *Wnt2* mutant combination demonstrate lower pigmented testes compared to wildtype; nevertheless, PCs were still present and the number was no different from the wildtype one, as detected by staining with an anti-Empty spiracles (Ems) antibody (Macias and Morata, 1996) which marks PCs (Nanda et al., 2009) (Fig. R. 17 C and F). Since we found in *Wnt2* mutants the expected wing phenotype, we inferred that the lack of internal phenotype in the reproductive system might be due to low penetrance (Kozopas et al., 1998; Nanda et al., 2009). However, in the case of *SOX100B* we did not have any other indication of the mutants being correct, as the gene is only required in the PCs.

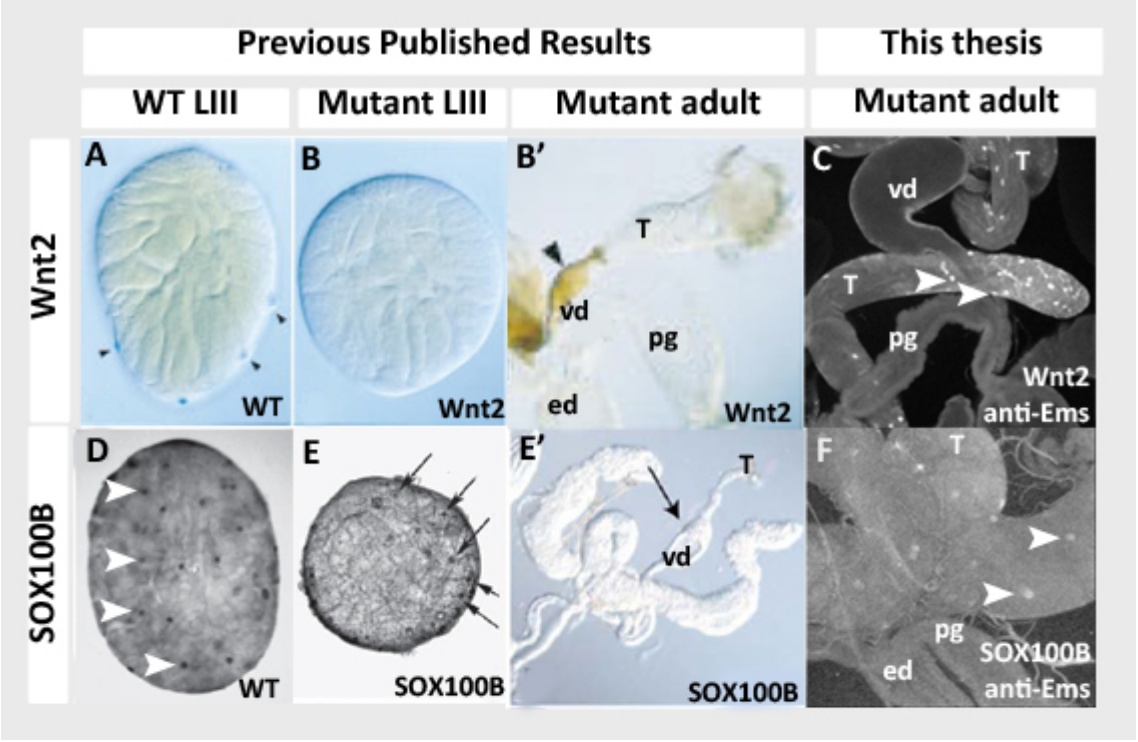


Fig. R. 17. Mutants with altered pigment cells, compared to Kozopas and Nanda results. A and B. Experiments by Kozopas et al., (1998). A Wildtype LIII testis where PCs are stained with 365 marker (in blue) (Gonczy et al., 1992). B, B'. Larval (B) and adult (B') testis of DWnt-2RJ/Df(2R)11 mutants. Note the irregular shape of testes and that vas deferens (indicated by a black arrow in B'), but no testes, are pigmented. C Our results. same Wnt2 mutant combination, testis displaying normal pigment cells, as shown by anti-Ems antibody staining (white arrow-

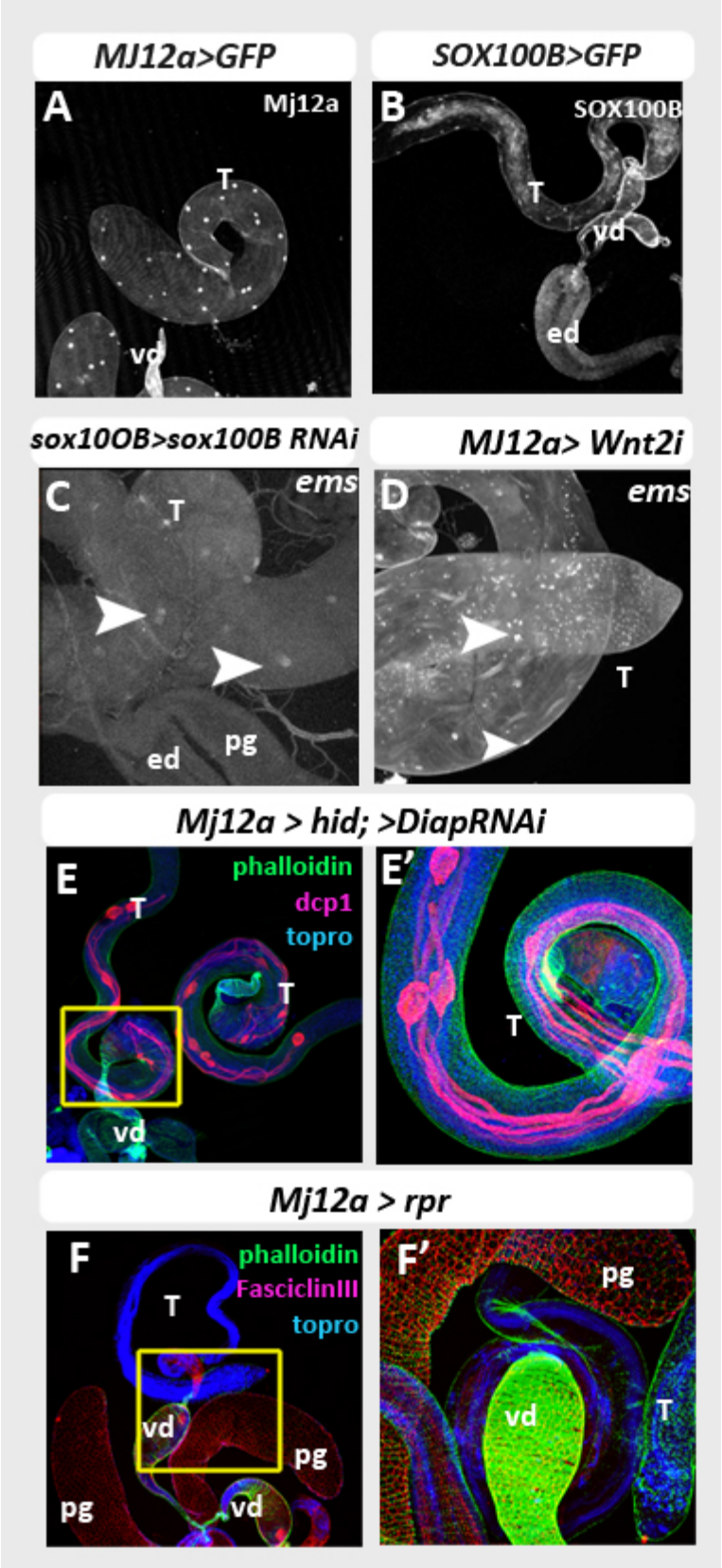
heads). D and E. Experiments by Nanda et al., 2009. Wildtype third instar larval testis (D) show a higher amount of pigment cells nuclei than sox100B (Df(3R)j3B9-rv12 and Df(3R)tll-g mutant ones; this is revealed by anti-Ems staining, shown as black dots in D (white arrowheads) and E (black arrows). This lower amount triggers major testis defects in the adult (E'). F Our results, with the same mutants used by Nanda et al., 2009, showed normal coiled testis (T) with normal pigment cells (anti-Ems staining, white arrowheads). Abbreviation as follows: T: Testis, vd: vas deferens, ed: ejaculatory duct, pg: paragonia,

We then tried to eliminate these cells using PCs-specific Gal4 lines and UAS-RNAi constructs against *SOX100B* and *Wnt2*. We used two such Gal4 lines: the *MJ12a*-Gal4 insertion (Hrdlicka et al., 2002) restricted to PCs (Fig. R. 18A) and *SOX100B*-Gal4, which shows a pattern of expression in tracheae of the genital disc and in PCs (Fig. R. 18 B). Reducing the expression of either *SOX100B* or *Wnt2* in PCs had no effect on testes coiling (Fig. R. 18 C and D); however, we observed that the gonads had not developed the characteristic yellow pigmentation. Confocal images demonstrated that pigment cells were present and that the muscular pattern was normal (arrowheads in C and D and not shown).

We also tried to induce PCs death by expressing the proapoptotic genes *head involution defective* (*hid*) and *reaper* (*rpr*) under the control of UAS sequences exclusively in PCs, but we did not obtain a major effect. In these experiments, *MJ12a*-Gal4 UAS-*hid* (Fig. R. 18E and E') and *MJ12a*-Gal4 UAS-*rpr* (Fig. R. 18F and F') testes would be white but normally coiled and we observed caspase activation only in in the sister spermatids, as previously, described(Kaplan et al., 2010) Fig. R. 16, Fig. R. 18E'), but not in PCs. Since PCs are in direct contact with the hub, where stem cells niche is located, we decided to check if the hub had been compromised by the activation of pro-apoptotic genes in the PCs. Our results proved that it was unaffected, as shown by Fasciclin III staining, which marks the hub (red dot in Fig. R. 18F') In summary, we have not been able to eliminate PCs and see the effect of such elimination in muscle specification or testis development.

Fig. R. 18. Genetic induction of apoptosis in the PCs had no effect on testis coiling and muscle structure.

A, B. The Mj12a-Gal4 line drives expression in pigment cells (white nuclei) (A), and so does the SOX100B-Gal4 line (B, white nuclei) C, D. Reduction of sox100B (C) or Wnt2 (D) expression does not affect testes coiling or eliminate PCs E-F' Induction of apoptosis by overexpression of hid and inhibition of Diap1 (E), triggers no PCs cell death (shown in more detail in E') although we did observe the normal apoptosis at the sister spermatids (anti-Dcp1 staining, marking dying cells, in red). The muscle actin pattern (phalloidin staining, in green) remained unaltered. F, F'. Similar results were observed when using UAS-rpr (F), and we observed that the hub region was normal (red dot, Fascilin III, in more detail in F'). TOPRO staining and abbreviations as in previous Figures.


MJ12a>GFP
SOX100B>GFP
A
Mj12a
T
vd
B
SOX100B
T
vd
ed
sox10OB>sox100B RNAi
MJ12a> Wnt2i
C
ems
T
ed
pg
D
ems
T
Mj12a > hid; >DiapRNAi
E
phalloidin
dcp1
topro
T
T
vd
E'
T
Mj12a > rpr
F
phalloidin
FasciclinIII
topro
T
vd
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pg
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F'
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T

R. 2. 5. 2 Pigment Cells numbers

Testis significantly increase their volume and surface from the third larval to the adult stage, and this led Nanda et al. (2009) to hypothesize that the number of PCs would double during metamorphosis. Besides, previous results (although we could not confirm them) showed that the reduction in the number of PCs in certain mutant combinations correlated with testis malformation and failure to coil (Kozopas et al., 1998; Nanda et al. 2009). This suggested a certain number of PCs, acquired during development, was needed for testes development. Therefore, we decided to analyze if there was indeed an increase in PCs number during pupal stages and if a minimum number of those cells was needed for testes elongation and coiling.

To study this, we have measured testis area and average area of PCs, and counted number of PCs at late third larval and adult stages. During third larval stage and early pupal development, PCs show an average number of 17±3 (n=5). By the end of pupal development, PCs are not only distributed on the surface of the testis but also on the vas deferens, and its total number has risen to 20 ± 2 in the adult (n=17) (Fig. R. 19 A). It seems clear that the PCs would not double their number to cover the surface of the testis during pupal stages. As there was such a small increase in the number of PCs, but the testis surface area greatly increases as they elongate, we wanted to know if PCs had increased their area. To check this, we measured both cell surface area and the distance between nuclei, as a proxy for increase of cell area, in third instar larvae and adults (Fig. R. 19 A).

As shown in the Figure (Fig. R. 19A), while PCs number remains more or less constant, with only a small increase from late larva to adult, the distance between pigment cell nuclei has a 3.4 fold increase and the pigment cell area a 3.9 one when comparing these two stages. Therefore, rather that doubling their number, as proposed by Nanda et al. (2009), PCs stretch to cover the testis, what (approximately) multiplies its surface by 5 (4.7 fold) from larva III to adult. The difference of the multiplying factor between testis area and PCs area may be explained by the small increase in the number of PCs in this developmental interval. Our next question was then to determine the origin of the new PCs.

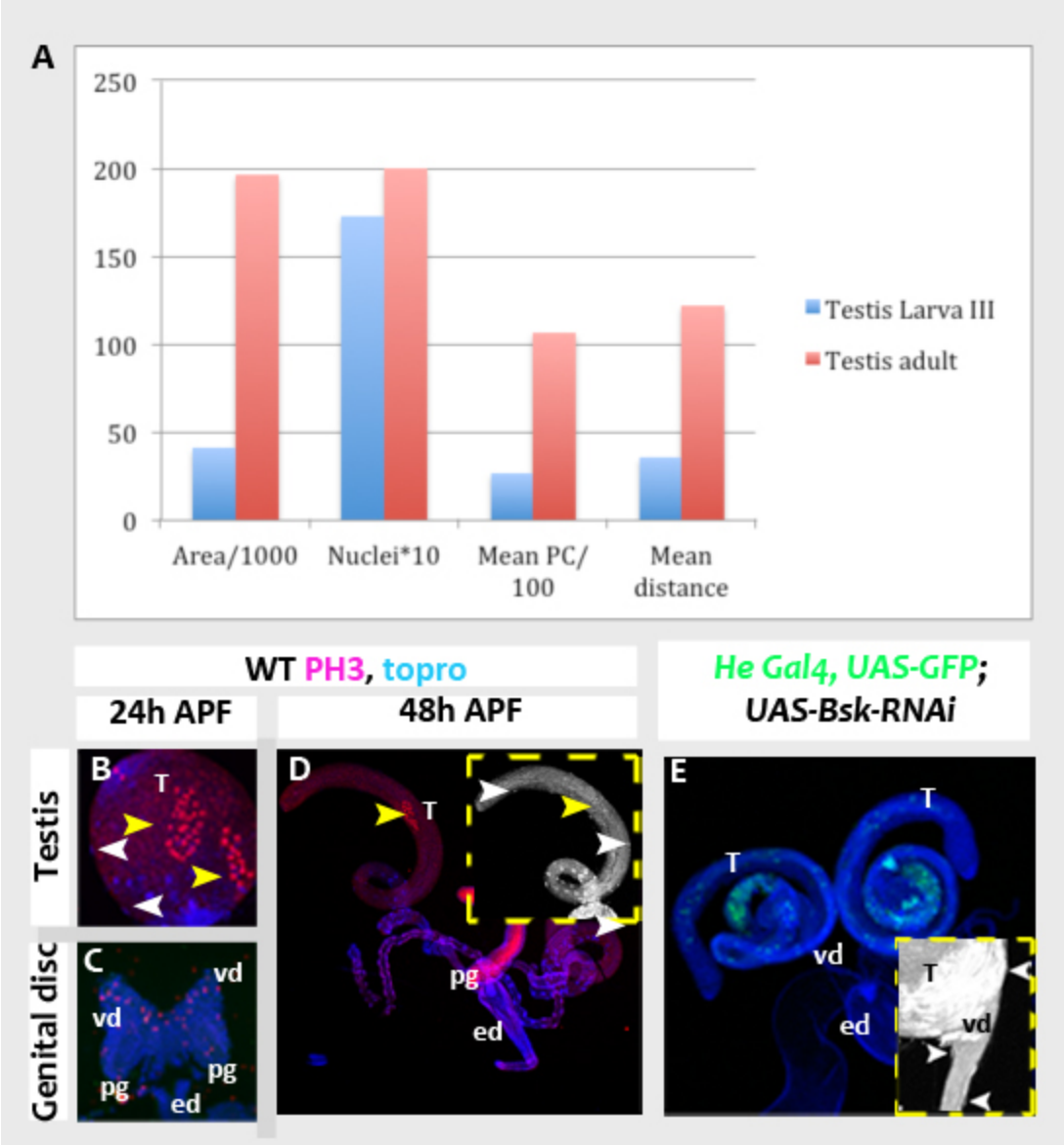


Fig. R. 19. The increase in PCs number after metamorphosis cannot be related to pigment cell division or migration from the fat body using the JNK pathway.

A. Differences in pigment cell number, mean distance among PCs nuclei and cell area in larva III stage (blue) and adult (red). B, C. Cell division, as recognized by anti-PH3 staining (red), in 24h APF testes (B, yellow arrows) and evaginating genital disc (C). Note there is no cell division in the PCs, on the surface of 24h APF testes (white arrows in B, point the PCs in the surface, marked with TOPRO) and that cell division in the disc is present in the leading edge of the vasa deferentia (vd). D. 48h APF testes, cell division is observed in spermatogonia cells (yellow arrows) but not in the surface or PCs (white arrows). E. Inhibiting JNK signaling in the fat body (He-Gal4 UAS-bsk RNAi mutants) does not impede the presence of PCs in the adult testis (white arrows in close-up) TOPRO is in blue.

R. 2.5.2.1 The origin of new Pigment Cells

Although the increase in PCs number observed when compared third instar larva and adult is small, we wondered if some cells will indeed divide during this period or if there was some cell recruitment. To check this, we determined, first, normal cell division in

gonads (Fig. R. 19 B) and genital discs (Fig. R. 19 C) during metamorphosis by staining with an anti-PH3 antibody, marking mitosis. We found cell divisions in the hub and in spermatogonia, giving rise to spermatozoids (Fig. R. 19 D yellow arrows), but no division in the testis outer layer, hence, no division in PCs (a close-up is shown in Fig. R. 19 D, dividing cells are indicated by yellow arrows, compare with absence of cell division in PCs, white arrows in B).

Although our results do not support the idea that PCs divide, we cannot exclude, however, an occasional cell division in pupal stages that will account for the small increase in the number of PCs. An alternative hypothesis is that the "new" PCs migrate from the fat body, similarly to the migration that takes place during gonad formation at embryonic stages (Whitworth et al. 2012). To test this hypothesis we inhibited migration from the fat body by depleting JNK or *matrix metalloprotease, 1* (*mmp1*) function (Pastor-Pareja et al., 2004; Cobbe et al., 2009; Glasheen et al., 2009; Glasheen et al., 2010), known to be needed for several migration processes, with a fat body Gal4 line, *He-Gal4*, (Pastor-Pareja and Xu, 2011), and UAS-*bsk* RNAi lines (*basket*, *bsk*, is a gene in the JNK activating cascade; Adachi-Yamada et al., 1999)). We found that testes would again lack pigmentation (in fresh dissections) but that their number seems to approach now that of the wildtype (Fig. R. 19 E and data not shown). Therefore, either there is an occasional cell division during metamorphosis, difficult to detect, the PCs were not migrating from the fat body, but were being recruited from elsewhere, or they would use alternative means of migration or signaling. This latter process, signaling in PCs, is studied in the next section.

R. 2. 5. 3 Signaling pathways in the Pigment Cells.

Similarly to what happens in testes muscles, we considered that signaling pathways could be required to achieve and/or maintain pigment cell fate and activity and to communicate with the muscle layer. That is why we used different signaling pathway reporters to establish the activity of the different routes in these cells.

R. 2.5.3.1 Role of the JNK pathway in the Pigment Cells and testis coiling

The JNK pathway is involved in many activities during development, (see section I. 5) Among them, in processes of migration and cell death, wound healing and, as recently described, in triggering active polymerization in muscles (Hong et al., 2014). By using the *puckered* (*puc*)-lacZ reporter (*puc* is both a target and an inhibitor of the pathway, Martín-Blanco et al., 1998) we were able to observe that JNK was active in PCs (Fig. R. 20 A).

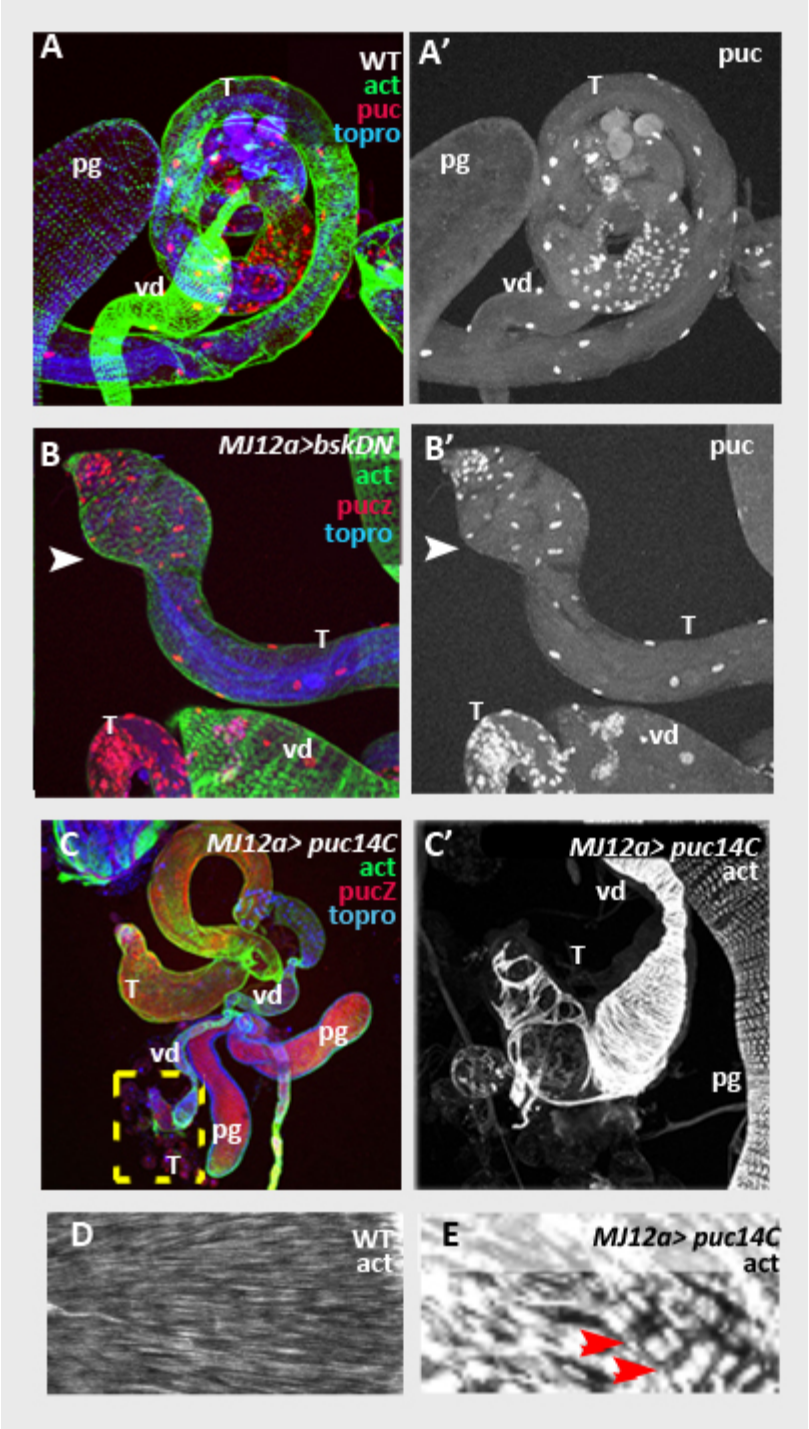


Fig. R. 20. JNK signaling pathway is active in the Pigment Cells and its depletion causes malformations in the testis due to malformations on the muscle.

A- A'. puc-lacZ is a readout of the JNK signaling pathway, and it is present in PCs nuclei (in red in A, in white in A'); muscles are marked by actin (in green) and nuclei by Topro (in blue) B. B, B' Alteration of JNK signaling, using MJ12a-Gal4 to drive expression of UAS-BskDN at the PCs, triggers testis malformations. C, C'. A dramatic testis reduction is observed when expressing UAS-puc14C at the PCs using MJ12a-Gal4 as a driver.D-D'. shows the area indicated in D in more detail). E. Muscle organization in MJ12a-Gal4 UAS-puc14C, mutants is not like that of the wildtype smooth muscle (compare with the WT muscle in (C). actin in green in A, B, D and in white in C, D' and E; puc in red in A and B, ems in red in D and TOPRO in blue. Abbreviations as in previous figures

To discover the possible function of the JNK pathway in pigment cells we used the *MJ12a*-Gal4 line, specific for the PCs, to reduce JNK activity by expressing UAS-*puc14C* (Kolahgar et al., 2011) or UAS-bsk^{DN} (Adachi-Yamada et al., 1999), two constructs which inhibit the pathway. We observed testis changes in morphology when using these lines, stronger and with more penetrance with UAS-*puc14C* (close to 100% penetrance). In *MJ12a*-Gal4 UAS-Bsk^{DN} flies the tip of the testis seems inflated (Fig. R. 20B, the wildtype in A), and when expressing the *puc14C* construct we observed that testis would be strongly reduced and muscles would not cover the full length of the testis (Fig. R. 20 D and shown with more detail in D'). Intriguingly, we also observed a change from smooth towards striated muscle morphology, as shown by muscular proteins organization (described in the section I. 4. 1) compare actin staining in the wildtype (Fig. R. 20 D) and mutant (Fig. R. 20E).

The JNK pathway is known to induce expression of pro-apoptotic genes in several contexts (Adachi-Yamada et al., 1999; Martín et al., 2009; Shlevkov and Morata, 2012). However, since these cells do not die (we have also previously demonstrated that expressing *hid* or *rpr* did not induce any phenotype in PCs, see Fig. R. 18) the role of JNK here might not be inducing apoptosis but, among other roles, allowing cell migration (PCs migrate over the vas deferens).

R. 2.5.3.2 Pigment Cells show activation of the JAK/ STAT pathway.

It is known that the JNK activates the JAK/STAT pathway, for example, to induce cell proliferation cell autonomously and non-cell autonomously (Katsuyama et al., 2015). We hypothesize therefore that JNK might activate JAK/STAT in PCs. The JAK/STAT pathway is associated with many biological processes that may be related to smooth muscle development. For example, JAK/STAT is needed for proliferation or Epidermal-Mesenchymal-Transition (EMT), a transformation produced in some differentiated epidermis that results in migration and some other characteristics of mesodermal cells. This pathway may also control the immune system; therefore, it can be associated with long-range signaling (Silver and Montell, 2001; Agaisse and Perrimon, 2004). In fact, it has been predicted as having one of the longest ranges of activity (20 cell diameters; Tsai and Sun, 2004) together with the Wnt pathway (25-30 cell diameters, Cadigan et al., 1998; Perrimon et al., 2012). If the JAK/STAT pathway was active in the PCs, its long-range signaling could help the male genital disc derivatives to reach the gonads and therefore allow muscle migration over the testis. Furthermore, the JAK/STAT pathway is activated after a septic wound and triggers differentiation of lamellocytes and crystal cells, which share several traits with PCs. The role of JAK/STAT in these processes, together with the prediction of this pathway triggering activation of muscle identity genes in tissues

adjacent to its place of expression (Nightingale et al., 2004), suggested it might be a good candidate for regulating testes shape, and thus worth to be studied.

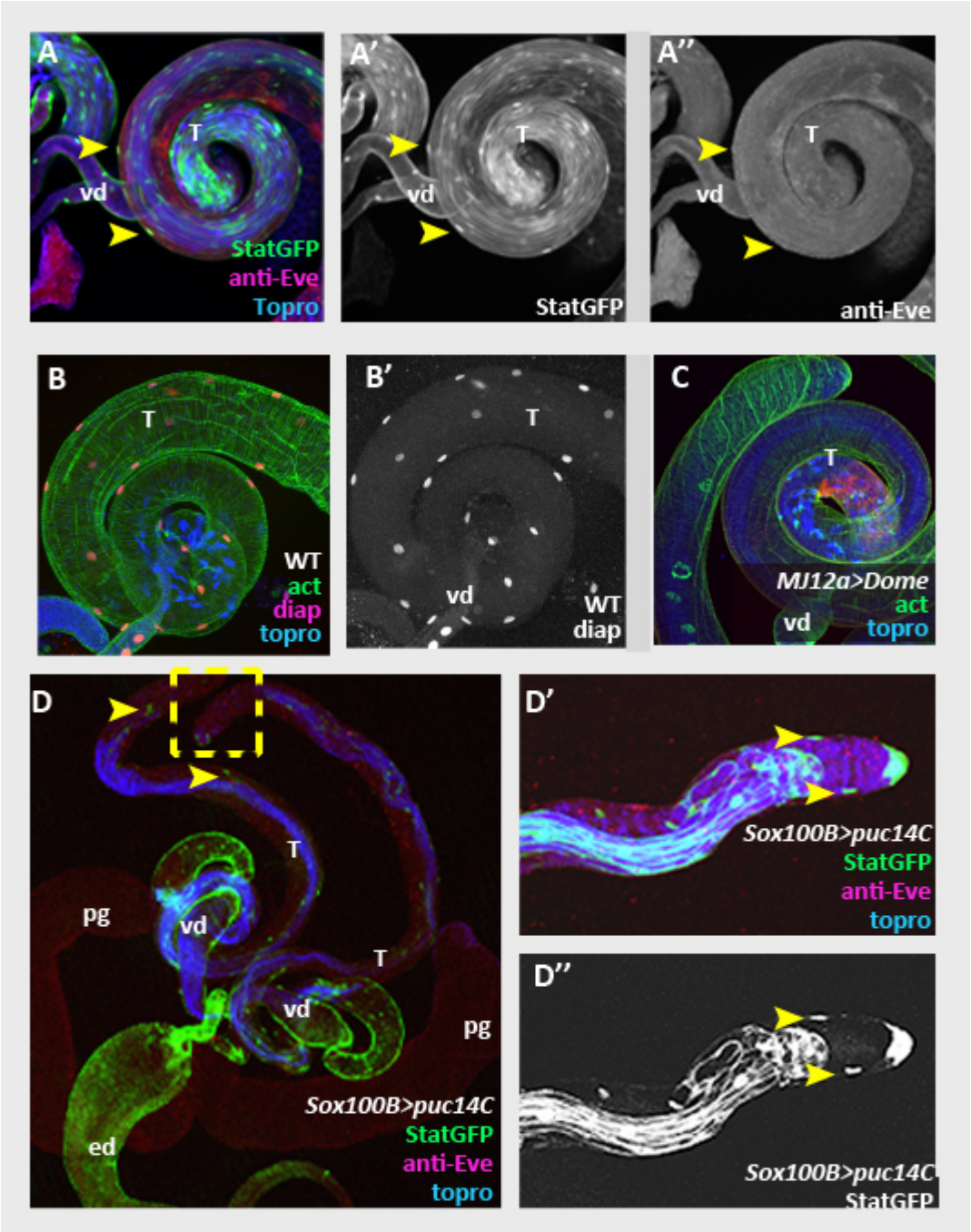


Fig. R. 21. JAK-STAT is also active in Pigment Cells but it does not depend on JNK pathway activity.

A-D'. PCs expression of STATGFP (in green in A, in white in A', arrows), Eve (in red in A , in white in A''), Ken and Barbie-lacZ (in red in B, larval testis, in green in C, in white in C', adult testis) and Diap1-lacZ (in red in D, in white in D'), targets of the JAK-STAT signaling pathway. E. Inhibition of the JAK-STAT pathway in the PCs (MJ12a-Gal4 UAS-Dome males) had no effect in testis coiling. act is shown in green. F-F". Inhibition of the JNK pathway (sox100B-Gal4 UAS-puc14C flies) did not decrease the levels of STATGFP or Eve (in green and red, respectively, in F, F'; STATGFP in white in F") in the PCs , of the marked region is shown in F' and F'').

We have observed that a member of the pathway, a reporter that reveals JAK-STAT activity, the Signal Transducer and Activator of Transcription-GFP (STAT-GFP) (Bach et al., 2007) (Fig. R. 21 A and A') and two direct targets, *even skipped* (*eve*) (Fig. R. 21 A and A'') and *ken and Barbie*-lacZ (*ken*-lacZ) (Castrillon et al., 1993) (Fig. R. 21 B and C, C') are actively transcribed in the PCs. This pathway also activates the transcription of *Drosophila Inhibitor of apoptosis-1* (*Diap-1)* (Betz et al., 2008), an inhibitor of the Drosophila effector caspase ICE (drICE) and *Drosophila Nedd2-like caspase (Dronc)* (Mills et al., 2005; Xu et al., 2006). In fact, we have observed with a *Diap-1* lacZ reporter that there is high *Diap1* expression in these cells (Fig. R. 21 D, D'). This high expression may account for the lack of effect observed when driving expression of *hid* in those cells (Fig. R. 18 F, F')*.*. If we wanted to induce cell death in PCs we should do it by activating the cell death pathway downstream Diap-1.

To ascertain the role of this pathway in PCs development we have tried to inhibit it by expressing RNAis against genes encoding the ligands Unpaired 2 (Upd-2) and 3 (Upd-3), the receptor Domeless (Dome) and STAT92, a nuclear effector of the pathway, with the PCs-specific *MJ12a*-Gal4 line, but little if any effect was seen on the testis and only a minor reduction of pigment was observed (Fig. R22 and data not shown). We have also assayed the activation of JAK-STAT by JNK. Inhibiting JNK in the PCs triggered no differences in the activation of JAK-STAT.(Fig. 21 F – F''). These results do not support a role for the JAK-STAT pathway in PCs.

R. 2.5.3.3 Pigment Cells change of sex.

As described in the Introduction, PCs are only present in males, forming the outer layer of the testis. The sex determination pathway has been explained in section I. 2. 1.; briefly, the *tra* gene is exclusively active in females and establish somatic female identity by directing the splicing of the *doublesex* (*dsx*) gene to the *Doublesex-female* (*Dsx-F*) form. In males there is no functional *tra* and *dsx* is spliced to the *Doublesex-male* (*DsxM*) protein. DsxM and DsxF expression are both revealed by the *dsx*-Gal4 construct, which is active since early LIII in the ovary (Fig. R. 23 B) or testis (Fig. R. 23 C).

We wondered if changing the sex of PCs in pupal stages would eliminate them (Fig. R. 23 E), as experiments with gynandromorphs showed that testis contacting female tissue would degenerate (Dobzhansky, 1930). To this aim, we forced the expression of *tra* with the PCs-specific (*MJ12a* and *SOX100B*) Gal4 lines and, to our surprise, we observed that PCs were still present (Fig. R. 23 F and a close-up is shown in F', compare to 23 A; the PCs are stained with anti-Ems antibody); perhaps even more striking, the muscle pattern

looked transformed from smooth to striated (Fig. R, 23 G, compare to Fig. R. 23 D, the wildtype pattern) and, less surprisingly, as a result, testes are malformed.

Therefore, testis muscle morphology responds to changes in their close environment (PCs JNK activity or sexual identity). This stimulated us to look carefully at the communication between the two tissues, and that is why we decided to look at the Extra Cellular Matrix (ECM) components and their possible impact in muscle specification and testes coiling.

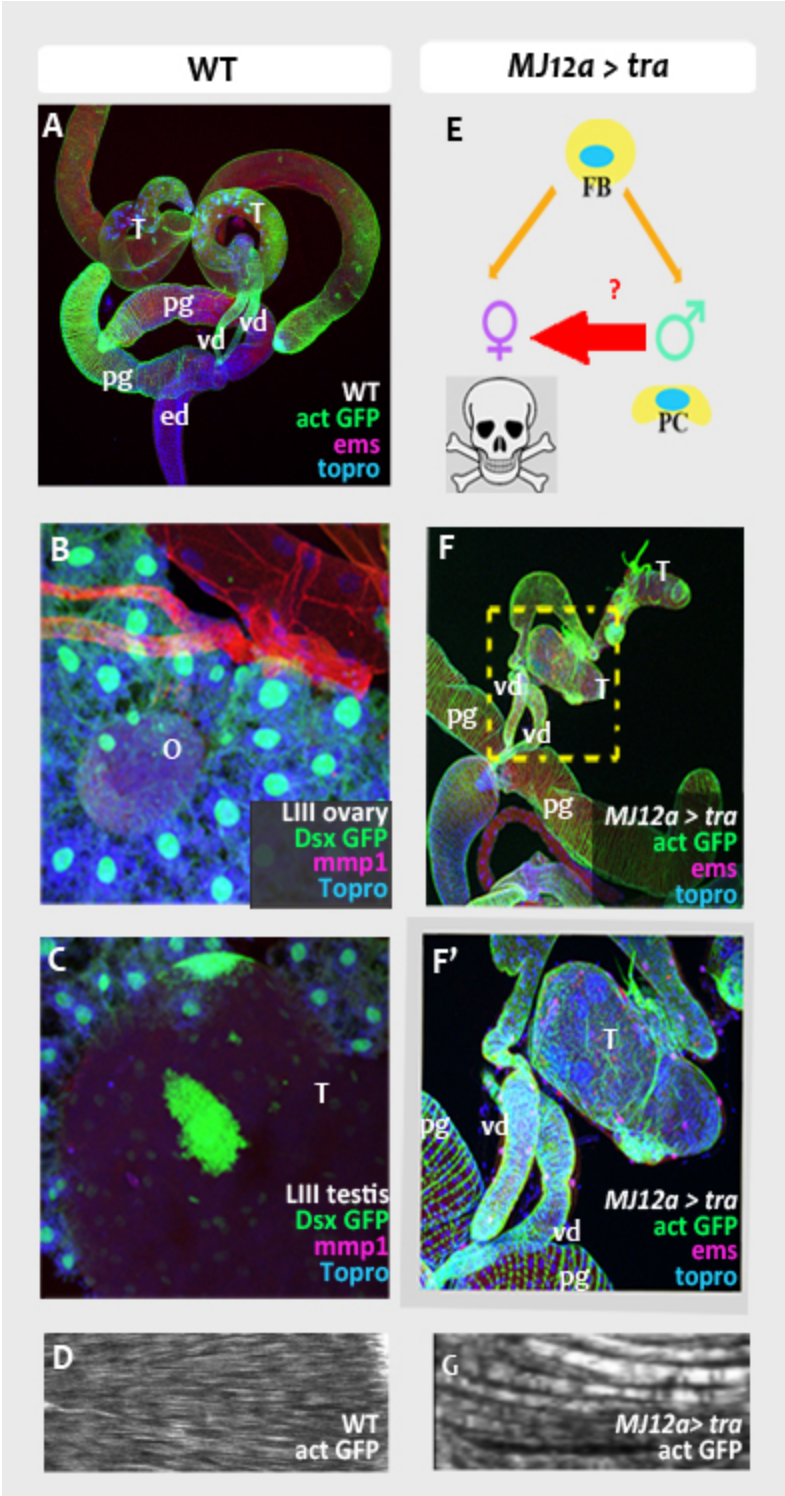


Fig. R. 23. Change of sex in Pigment Cells causes malformation in testes
A. Adult wildtype, muscles identified in green (actin-GFP)and PCs in red (anti-Ems antibody) B. Larval ovary (O) showing dsx-GFP expression (in green) and mmp1 (in red). C Larval testis (T) showing Dsx-GFP expression (in green) and mmp1 (in red). D. Actin smooth muscle pattern. E. Scheme representing the expected cell death of PCs after their change of sex. F. MJ12a-Gal4 UAS-tra mutant flies, where the vas deferens contacts the gonad but testis do not elongate and coil normally; a close up (F') shows that the double migration is produced, as there are muscles (in green, actin) over the testis and PCs (in red, anti-Ems) over the vas deferens. There is a change in sarcomere organization in the mutant, compare D to G (actin striated muscle pattern). TOPRO in blue. Abbreviations as follows: O: Ovary. T: Testis, vd: vas deferens, ed: ejaculatory duct, pg: paragonies, sp: spermatic pump, An: analia

Therefore, testis muscle morphology responds to changes in their close environment (PCs JNK activity or sexual identity). This stimulated us to look carefully at the communication between the two tissues, and that is why we decided to look at the Extra Cellular Matrix (ECM) components and their possible impact in muscle specification and testes coiling.

R. 2. 6 ECM and testis development

Although it has been demonstrated that testis shape changes after they are contacted by the vas deferens (Stern and Hadorn, 1939, Fig. R. 7), D'Ávila et al. (2008) described that *blistered* (*bs*) *Drosophila willistoni* mutants had alterations in testis shape while attached to the vas deferens. *blistered* is a gene required for vein formation and whose mutants present blisters in their wings. Blisters formation is related to a mis-localization of ECM components, such as integrins or laminins (Fristrom et al., 1994). This suggested that components of the ECM might be required to shape testis. Pastor-Pareja and Xu (2011) demonstrated that the proteins encoded by *viking* (*vkg*) and *terrifically reduced optic lobes* (*trol*) (Collagen IV and Perlecan Drosophila orthologs, respectively), secreted by the fat body, are needed for the correct elongation of the neural cord and for a normal shape in the wing imaginal disc; similarly, it was also demonstrated that the ECM is a key factor during oogenesis, allowing the egg to attain its correct shape (Gates, 2012; Haigo and Bilder, 2011; Bastock and St Johnston, 2011; He et al., 2011). All these data suggest that ECM components may have a role in determining the shape of testes.

Most of the components of the ECM are produced by every cell, excepting Collagen IV and Perlecan, which are made by mesodermal cells in the fat body. The asymmetrical distribution of the ECM components allows cell polarization in any tissue. In the basal area, outside the cell, we can find collagen IV, encoded by the gene *vkg*, which helps cell stretching (Pastor-Pareja and Xu, 2011). Vkg is in direct contact with Laminins, specifically Laminin A, which in turns contacts with Perlecan. Perlecan, in turn, makes direct contact with the dystroglycans and directs their position, what is determinant for muscle viability and laminin localization (Graner et al., 1994; Deng et al., 2003).

On the baso-lateral side of the cell membrane we can find the septate junctions, which are specific areas where integrins from two neighbor cells interact. As described before, integrins are key players in the communication between cells and with their environment (Schotman et al., 2009). They are transmembrane proteins that once activated in the outside of the cell may trigger signaling cascades to the inside ending in the transcription of specific genes. In Drosophila, integrins are composed of two different α-subunits, Inflated (If) and Multiple edematous wings (Mew) and a ß-subunit, Myospheroid (Mys).

Integrins contact in the outside with Laminin B, Trombospondin (Tsp) and Tiggrin (Tig), but also with Collagen IV, and therefore Laminin A and Perlecan (Gutzeit et al., 1991; Davies, 2001). In the inside of the cell integrins contact with Talin (encoded by the gene *rhea*), and two kinases, Integrin related kinase (Ilk) and F-actin Kinase (Fak), which are in contact with the actin cytoskeleton (Bateman et al., 2001; Davies 2001). This actin cytoskeleton is a basic feature in muscles; therefore, it is interesting to know how its function is regulated. Since, as explained above, changes in the activity of ECM proteins have been shown to affect organ shape, we studied if a correct ECM function is also needed for normal testis morphology.

R. 2. 6. 1 Role of ECM in Pigment Cells development.

PCs derive from the fat body, and may retain therefore some ability to produce Collagen IV and Perlecan, which have an antagonistic effect in organ shape (Pastor-Pareja and Xu, 2011). In the testis, Collagen IV accumulates at the junction of muscle cells and the basal membrane that enclose the germ line and around the PCs nuclei (Fig. R. 24 A, A'), indicated with yellow arrows). This may suggest that PCs might produce Collagen IV and retain some fat body features. We have tried to inhibit *trol* in PCs using a UAS-*trol* RNAi line driven by the *MJ12A*-Gal4 driver and found no significant phenotypes (Fig. R. 24 B) but it is possible that, even though PCs may synthesize its own Perlecan, the Trol protein produced by the fat body may be enough to shape testes. However, we have found an abnormal accumulation of Tropomyosin at the tip of the testis (Fig. R. 24 B', yellow arrow), indicating that testis muscles are either in the first step of turning into striated muscles or having attachment problems, either to among the fibers or to the PCs. This effect may indicate the need of a communication between muscles and PCs through the basal membrane. We have also tried to inhibit normal transmission from the outside of the cells to the inside by inhibiting *laminin A* and *talin* (*rhea*) expression. When we inhibited *laminin A* at the PCs (*MJ12a*-Gal4 UAS-*lamA* RNAi) we observed asymmetrical phenotypes (Fig. R. 24 C): while one testis would not attach to the vas deferens, the other one would be attached to both vasa deferentia and displayed a Y shape (Fig. R. 24 D, the yellow arrow points towards the Y shape). This suggests there is in the mutant combination an inefficient communication between testis and vas deferens through the ECM. The phenotype observed when inhibiting *talin* at the PCs show vasa deferentia unable to attach to the testis (Fig. R. 24 E), and even when they do there are some testis defects, the hub would be constricted and looked separated from the full length coiled testis (Fig. R. 24 F, note that yellow arrow points to the constriction at the tip of the testis).

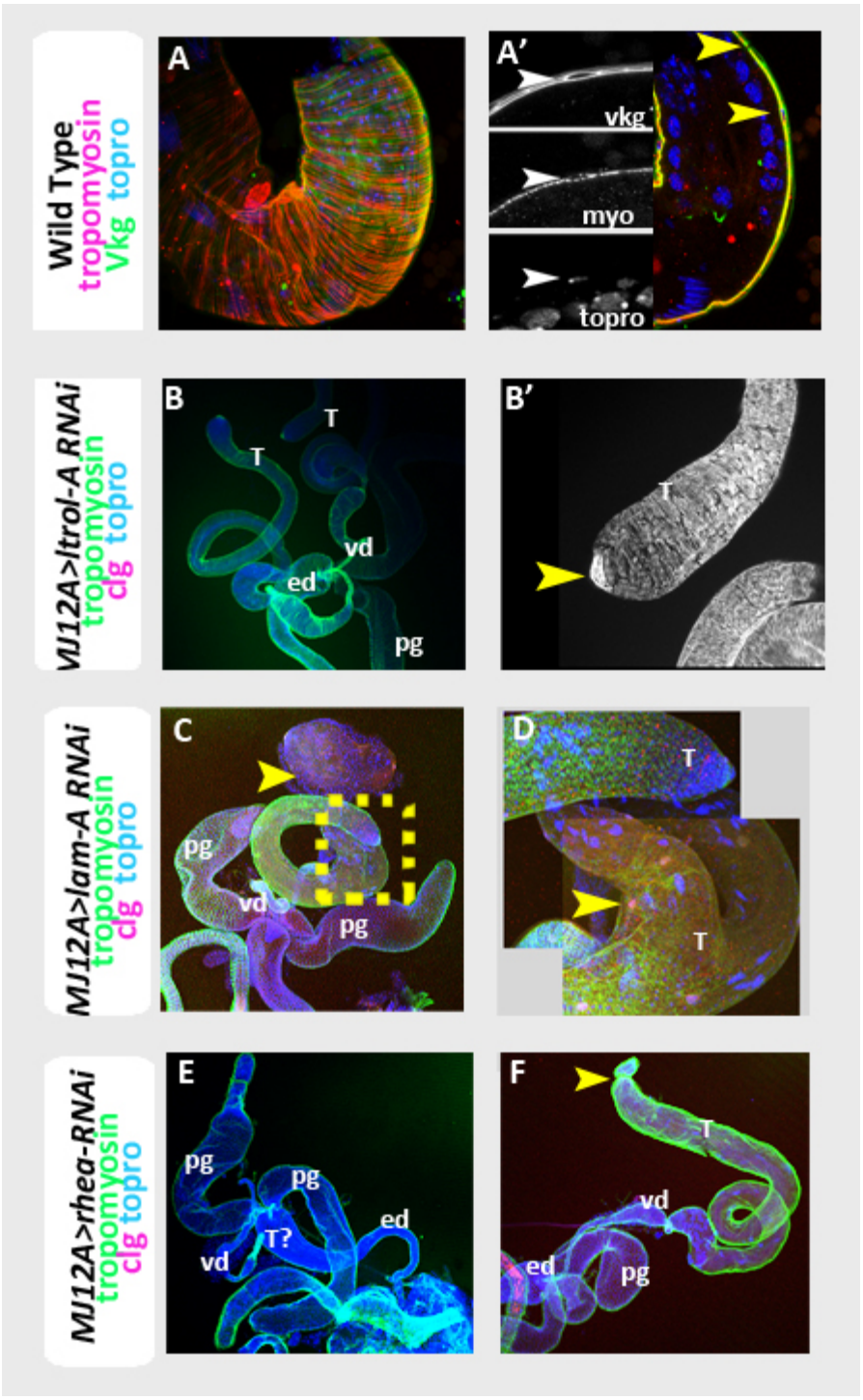


Fig. R. 24. Depletion of some ECM components in the Pigment Cells causes defects in testes development.
A. In the testes, Vkg (in green) is distributed in an outer layer and marks the limits of muscle cells (marked in red by tropomysin); in a optical section of the same testis (A') PCs nucleus may be observed as a discontinuity of the Vkg layer (yellow arrows in A' point to some of those discontinuities)A magnification is shown in the insets. B, B'. Inhibition of trol does not cause any major change in testis structure; we observed only an accumulation of tropomyosin at the hub of the testis (arrow in B'). C. Inhibition of laminin A specifically in the pigment cells trigger aberrant attachment of vas deferens and testis. Note the free testis (yellow arrow in C) and the two vasa deferentia contacting the same testis that, in turn, divides in two (shown in detail in C'). D. Inhibition of talin by expressing rhea-RNAi inhibited vas deferens attachment to the testis (missing testis is indicated with T?) or showed minor defects in testis: note, for example, the constriction at the hub of the testis (E).. Abbreviation as follows: T: Testis, vd: vas deferens, ed: ejaculatory duct, pg: paragonia.

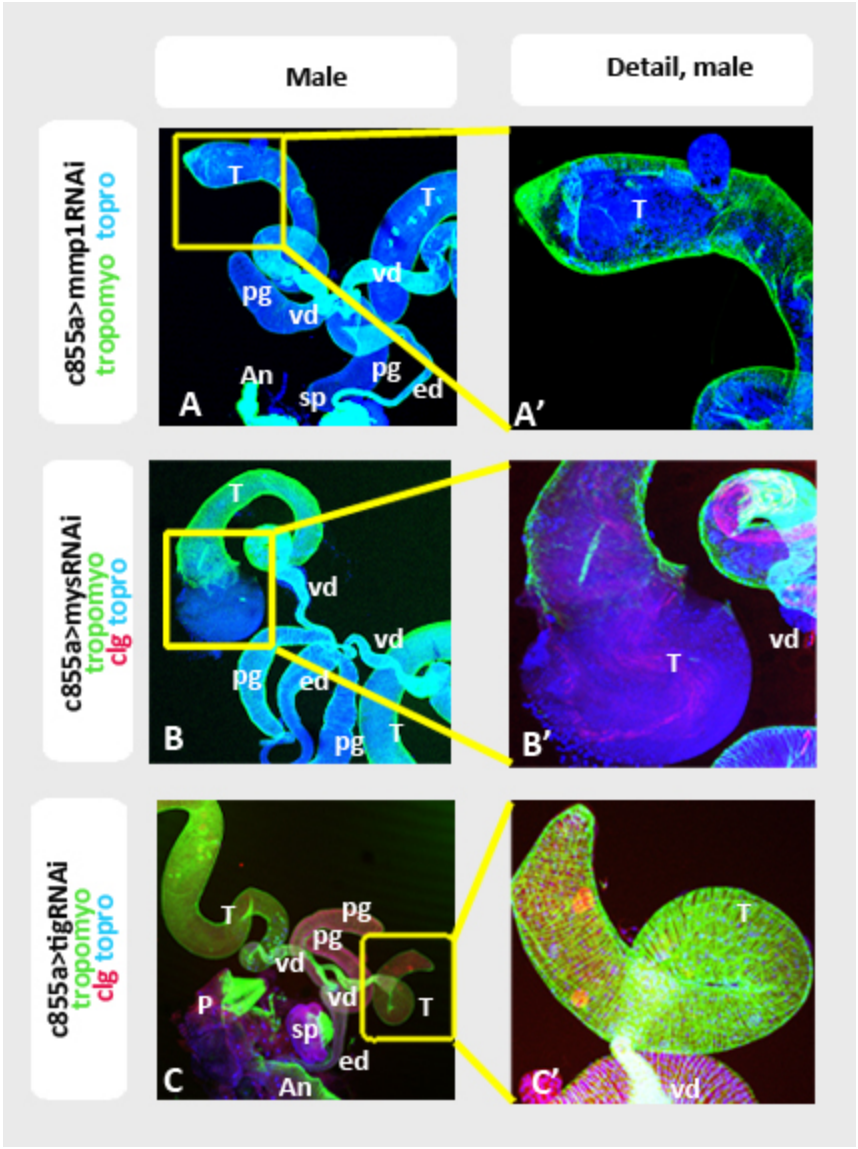
R. 2. 6. 2 Role of ECM in smooth muscle development.

The role of ECM in muscles is crucial, as the localization of actin, one of the main proteins of the sarcomere, depends on integrins (Bateman et al., 2001); besides, the strength of the

muscle would be determined by Myosin II (Gates, 2012), whose assembling also depends on Talin or Fak. Higher concentration of the former is related to higher concentration of Myosin and lower of Actin, thus triggering lengthening, a phenotype similar to gain of function *vkg* or loss-of-function *trol* mutants (Gates, 2012; Pastor-Pareja and Xu, 2011). These two proteins, Actin and Myosin, are key to define a muscle as smooth or striated, and for this reason it is interesting to study how the ECM components are distributed in Drosophila testis.

Fig. R. 25. Depletion of some ECM components in the muscle cells causes testes malformations.

A. Depletion of mmp1 (in red) in the testis muscle (c855a-Gal4 UAS-mmp1 RNAi flies) resulted in normal morphology and only a minor phenotype, a strong concentration of tropomyosin at the tip of the testis (in green; shown in detail in A'). B. Inhibition of the integrin ß-subunit Mys specifically in the muscle cells (c855a-Gal4 UAS-mys1 RNAi mutants) triggered changes in testis morphology: the tip of the testis was ballooned and the elongation of the testis correlated with the limit of muscle migration (shown in green with anti-Tropomyosin staining; a close-up is shown in B'). C. Inhibition of tiggrin provoked asymmetrical phenotypes: observe that one testis is smaller than the other, shown in detail in (C').clg: collagen, in red (B, B', C and C').topro in blue Abbreviation as follows: T: Testis, vd: vas deferens, ed: ejaculatory duct, pg: paragonia, sp: spermatic pump,



We have performed some gene depletion experiments in order to know which of the ECM components may be important to understand the difference between smooth and striated

muscles. But we wanted first to know if muscles needed to degrade the ECM of the PCs while migrating, and we have found that when we inhibit the *mmp1* gene only minor phenotypes are observed (Fig. R. 25 A, and shown in more detailed in A'),: the tip of the testis might seem a little inflated and has an accumulation of actin at the hub region, which is intriguing as there should be no muscles at this region. This effect which remind us, nevertheless, of the phenotypes observed when *trol* expression was reduced in PCs (see Fig. R. 24 B and B')..

Inhibition of the ß-subunit Mys in the muscle resulted in problems in muscle attachments (among muscle fibers and to the PCs) that relate to malformations in the testis (Fig. R. 25 B and B'): testis elongate and coil normally up to the limit of muscle migration, but not beyond, this limit marking the beginning of a rounded shape at the tip of the testis (Fig. R. 25 B and in more detail in B'). This is similar to the phenotype observed when we inhibit muscle division during pupal stages (see Fig. R. 7). We reasoned that, if the phenotype observed in males was due to the lack of muscle attachment, some proteins involved in cell adhesion should have a similar phenotype. That was why we studied the effect of reducing *tiggrin* expression. These mutants showed asymmetrical phenotypes, where one of the testes was considerably smaller than the other and less elongated but coiled (Fig. R. 25 C and shown in more detail in C'). We could not detect, however, any change in the sarcomere organization of the muscles (and therefore, in specifying smooth muscle) (Fig. R. 25C').

R. 3 Role of testis coiling in fertility.

Different species of *Drosophila* show different degrees of coiling: for example, *D pseudoscura* testes describe half a loop while *D. virilis* present testes with more than 4 loops. Joly and Schiffer (2010) related testis length with sperm length, concluding that the longer the sperm the longer the testis. Coiling has been related with spermatogenesis (Beall et al., 2007) and whether this coiling is required for fertility or not was addressed by Woolf (1965), who considered that morphology was not related to fertility, as he observed motile sperm in unattached testis. Dobzhansky and Beadle (1936) also found motile sperm in *D. pseudoscura* unattached transplanted testis; however, this species has uncoiled testes.

As previously demonstrated, coiling of the testes is produced only after attachment to the genital discs derivatives (Stern and Hadorn, 1939; Dobzhansky and Beadle, 1936). As we

have demonstrated, muscle migration is accompanied by testis morphological change and may be the force directing it. Thus, having shown several genotypes with incomplete testis development. we found it interesting to study if there was a minimum coiled shape required for fertility.

We have analyzed genotypes with incomplete coiling of testes (and less pigmentation), and found only a 18% of infertility. We first tested the infertility and later dissected individual males. Those males then were categorized in the following classes: a) aberrant morphology (Fig. R. 26 red sector), which included abnormally elongated or coiled testis (but not gonads absence or unattached gonads, which would render reproduction impossible), b) lack of pigment in PCs (Fig. R. 26, green sector), and c) both characteristics, when they lacked pigmentation and had not fully normal morphology (Fig. R. 26, purple sector). We found that the infertility is morphology-related in 61% of the cases, while in 24% of the total it is related to PCs anomalies and in 14% of the cases combines the two previous factors (Fig. R. 26 C and D, the wildtype in B).

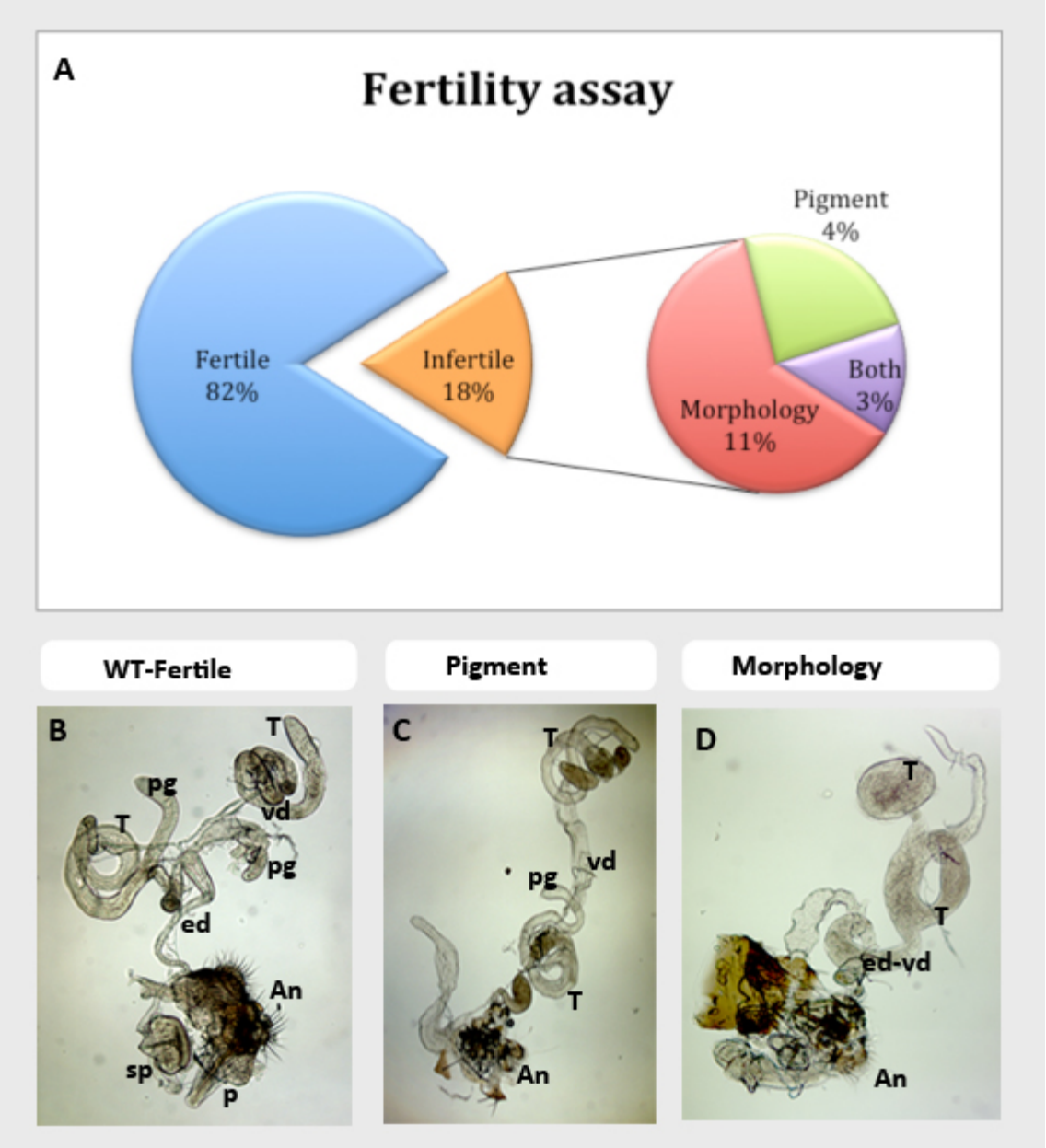


Fig. R. 26. Fertility analysis related to the internal genitalia mutant phenotypes.
A Diagram showing the percentage of fertile and infertile males (n= 128) and, within the latter, those due to changes in morphology, pigmentation or both. **B-D** Images of the two mutant classes are shown in C and D, and the wildtype in B.

DISCUSSION

In this work we have tried to understand, how the adult male reproductive system of Drosophila develops, and have found that this requires signalling events between different tissues to allow the contact between gonads and genital disc (Dobzhansky and Beadle, 1936; Stern and Hadorn, 1939a; Pantelouris, 1955; Babcock, 1971b). Besides, signalling from pigment cells, a group of cells surrounding testis muscle, to this tissue, is fundamental for the coiling of the testes. We have related these two main processes with changes in fertility, which we will discuss in this section.

D. 1 The precise contact between genital disc and testis required for the correct formation of the reproductive system

Metamorphosis triggered by specific hormones produces radical changes in Drosophila body conformation. In the reproductive system, those signals cause the evagination of the genital disc towards the gonads and a small elongation of the gonad toward the genital disc. However, the long distance that the genital disc must cover to contact the gonad challenges the idea of its being able to direct its evagination without any cues, such as environmental positional cues, similarly to the non-autonomous determination of muscles (Figure I. 9).

To explain how derivatives of the genital disc migrate in the right direction to contact the gonads in males and females it has been long argued that there should be a signal directing genital disc evagination (Stern and Hadorn, 1939b; Babcock, 1971a; Kozopas et al., 1998). Our results support the hypothesis that genital disc derivatives form in the absence of gonads but that gonads (particularly testes) do not properly develop during pupa in the absence of genital disc (Fig. R. 1, 2, 5); morphological changes may be appreciated in both cases. In females, unattached oviducts modify their structure, as previously described (R. 2 and 3; Babcock, 1971b) and we have found that they end up with nervous tissue attached to the seminal receptacle. The folding back of the oviducts towards the seminal receptacle was not previously described. It is possible that, in experiments where gonads were surgically removed, some nerves or nervous cues may remain surrounding the ovaries; in our experiments, the absence of such cues would drive oviducts to attach to an innervated structure, the seminal receptacle, which is the entry of the nerves that will innervate the oviducts (Fig. R. 3). In males, unattached testis failed to elongate and coil. This failing,

observed in the absence of the genital disc, agrees with previous observations (Stern and Hadorn, 1939b) and will be discussed in subheading D2.

During pupation, larval tissues disappear to give rise to the imaginal ones; however, the position of the latter may be determined or guided by some other tissues, similar to what happens in muscle migration, which uses the tracheal system as a guiding cue, or the Malpighian tubes, being directed by haemocytes (Ainsworth et al., 2000; Bunt et al., 2010; Montell, 2006; Myat, 2005). We have tested the role of the midgut and hindgut, as a guiding or supporting tissue for genital disc evagination because it is a tissue close to the gonads and genital disc, and because the genital disc evaginates from posterior towards anterior while the midgut migrates from anterior towards posterior a little time before. However, the mutants and genetic combinations assayed did not give the results we expected, and therefore prevented us from concluding about its impact on this process (Fig. R. 6).

Regarding the possible positional clues, there are two tissues that may provide the necessary information: ectoderm and fat body, both of them expressing Hox-genes. On the one hand, the ectoderm provides the regional cues for muscle specification (Dutta, et al., 2004; Dutta and VijayRaghavan, 2006); however, disruptions in the specification of ectodermal cells, as one could expect to occur in $abdA^{iab4}$ mutants (although this mutant would change just the A4 into the A3), had no effect in the evagination of the genital disc. Therefore, the ectoderm might not be the tissue providing the positional information required. On the other hand, the role of the fat body as a positional cue has not been yet analysed.

The fat body is a dynamic tissue involved in many functions, such as nutrient storage (Arrese and Soulages, 2010); it produces collagen IV and perlecan, as well as hormones and other long-distance products required for signalling (Lazareva et al., 2007). In this sense, it is also interesting to note that Hox genes are expressed in the fat body (Marchetti et al., 2003; Banreti, et al., 2014). Moreover, the fat body gives rise to different tissues, such as the haemocytes, crystal cells and PCs, that may retain some of its characteristics. As explained before, haemocytes help Malpighian tubule cells to find their path (Ainsworth et al., 2000; Bunt et al., 2010; Montell, 2006); therefore, it is possible that either the fat body or the tissues derived from it may provide information to the genital disc to help it evaginate towards the gonads. However, Hox gene expression has been shown to be down-regulated in late third instar larvae (Banreti et al., 2014) so, if this tissue is providing information, either Hox gene expression is resumed in pupa, at the time of genital disc evagination, or Hox genes are not the key guiding cues.

Depletion of ECM components in PCs and muscles, which may be made by the cells themselves or obtained from the fat body, suggest that the ECM might be involved in the two processes of tissue contact and recognition: first between gonads and genital disc and later between muscle and pigment cells. The deposition of ECM components produced by the fat body depends on the information that each cell provides through integrins and laminins (Urbano et al., 2009). Depleting integrins in the testis muscle results in aberrant testis morphology (they fail to elongate correctly, Fig. R. 22), while reducing laminins expression in the PCs cause aberrant contact and/or recognition between gonad and genital disc (one gonad remains unattached while the other divides in two, Fig. R. 23),. However, down-regulating *trol* (coding for the protein Perlecan) in the fat body only triggered minor phenotypes, not related to attachment but to constriction (Fig. R. 21) (although we do not know to which extent *trol* RNAi reduces *trol* expression). This suggest that ECM may not be crucial for the long range signalling but may have a leading role in the recognition and attachment of muscles during their migration under the PCs.

Regarding the relation between correct communication among different tissues and fertility, it is obvious that when there is no contact between gonads and genital disc, adults will be infertile due to mechanical reasons. However, there are cases where weak phenotypes are obtained, in which the contact was achieved but the testis fail to elongate and coil normally; these cases are a good model to understand how complete interaction and organ development (in this case, coiling) may be required for fertility. We will discuss this point in what follows.

D. 2 Coiled testis and fertility

During metamorphosis, testes undergo a four-fold increase in surface and a ten-fold increase of volumen. Testis and sperm length overpass by two to three-fold the total length of the *Drosophila melanogaster* body, and therefore, in order to accommodate inside the abdominal cavity both have to fold or coil. Coiled structures are found widely in nature, from tendrils till galaxies, from snail shells to the disposition of the leaves and flowers (phyllotaxis), allowing maximum exploitation of light in the minimum space. Connected to the Golden Ratio, the Fibonacci sequence applied to coiling allows a shape that encloses maximum area for minimum edge (Douady and Couder, 1992; Douady and Couder, 1993;) and therefore allows maximum growth while occupying the minimum volume. This may be the reason why testes in Drosophila coil after elongation, once they reach the maximum allowed length inside the body cavity (Fig. I. 7; Epper, 1983); they need to coil to fit in and allow normal spermatogenesis.

Previous researches (Castrillon et al., 1993; Grice and Liu, 2011) have demonstrated that there is a tight correlation between volume of the body and length of the testis, and between testis length and fertility. First, the bigger body volume a species has, the longer the testis may be (they have more room to fit inside); and the longer a testis is, the higher the length a spermatozoid can reach; finally, the longer the spermatozoid is, the longer it can survive in the female seminal storages, thus allowing an extended or postponed fecundation and therefore increasing the species fertility. This has been studied in different species of Drosophila, the main conclusions being that sperm and testis length are closely related and that both depend on body mass (Pitnick, 1996; Pitnick et al., 1999; Joly et al., 1997; Schärer et al., 2008). In accordance with this, Beall et al. (2007) demonstrated that size of spermatozoids and spermatogenesis determined testis size and Joly et al., (1997) observed infertility problems due to an abnormal spermatogenesis related to a shortening or reduced length in the testis.

To sum up, *Drosophila melanogaster* displays long coiled testes that produce long sperm. But there are some species that have shorter sperm and whose testes are not even coiled; nevertheless, they are fertile. A possible explanation is that having shorter sperm may also have advantages, because this requires less energy to be produced. Some species may have a long flagellum, which has some advantages for embryos, as will be discussed in what follows. Flagella are made by tubulin, and therefore the longer the tail of the sperm, the greater the amount of tubulin. This vast quantity of tubulin would allow faster divisions of the zygote, as cells would not require to synthesize tubulin anew (Lattao et al., 2012).

Apart from the higher costs of producing longer tails, the size of the sperm has some other advantages. The size of the sperm forces its coiling: it displays a double helix structure, similar to a corkscrew, that is related to a double wave movement, what implies that a small energy input is translated into a big force. In our system, the balance between forces of propulsion in the sperm and contention by the testes wall has to be neutral, otherwise either the sperm would not be able to reach the exterior or the testis would be punctured by millions of tiny corkscrews (Pak et al., 2012). Therefore, how do males and females prevent the punctures made by sperm?

While studying species with two different sperm length, Bressac et al. (1991) found that long sperm changed their coiled shape inside the seminal receptacle of the female, remaining motionless till it was once again needed. Females could induce motion in the sperm by a strong muscle contraction at the seminal receptacle or spermatheca. These facts allow us to draw two conclusions: first, longer sperm are selected to allow a delayed

fertilization, which may help in harsh environments with little food or lack of a suitable place to lay eggs; and second, and more relevant to our study, that muscles may trigger changes in sperm motility and therefore, fertility differences. If this were true, smooth muscles in the testis would maintain sperm motionless until copula. At that moment, striated muscles in the vas deferens strongly contract, thus activating the sperm. Once motile, sperm abandon the testis and vas deferens towards the ejaculatory duct and are again boosted by the spermatic pump towards the female uterus. Conversely, having a more compact striated structure may lead to stronger contractions that might trigger earlier motility, or else, a more compact structure of muscles may be related to shorter testis, related then with the infertility cases we have observed. If these assumptions are correct, they may explain why the muscle surrounding the testis is the only smooth muscle in Drosophila.

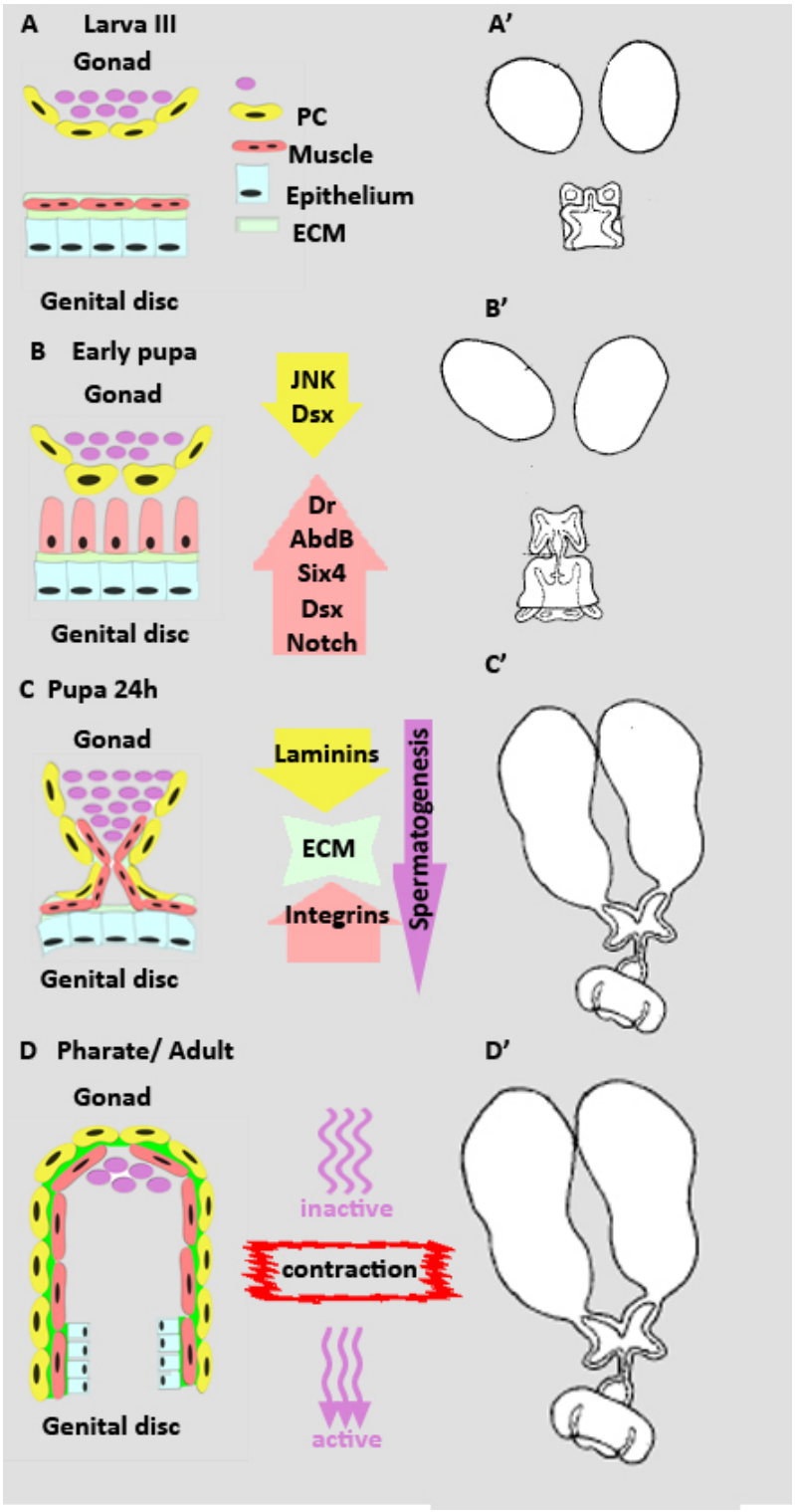
Mechanistically, the coiling of the testis might be due to the differential excretion of membrane from left and right sides of the testis (Miller, 1941), and functionally, the change of shape of the testes might be due to two simultaneous events: first, division of gonadal stem cells (GSCs), that forces the testis to grow, and second, division and contraction of muscles coming from the genital disc, which may softly push the GSCs anteriorly, forcing the elongation of the testis. Since transplantation experiments have shown that an uncoiled gonad would coil when contacted by a genital disc from a species that coil testes (Fig. I. 6 B and B'; Dobzhansky and Beadle, 1936; Dobzhansky and Beadle, 1936; Stern and Hadorn, 1939a) we have been more interested in understanding the importance of the interaction between pigment cells from the gonads and muscles from the genital disc, as we will discuss in D3.

D. 3 Coordination between muscle type and Pigment Cells during the development of coiled testis in *Drosophila melanogaster*.

The mechanism behind the elongation and coiling of the testis has been addressed in this thesis at different levels and from different points of view. We have studied the genetics and signalling pathways behind the tissues involved and the role that the ECM might play in this process. Knowing the importance of the contact between testes and genital disc for the change of shape of the former, we looked at the components of the gonads and the genital disc that may have a role in testes elongation and coiling.

Fig. D. 1. Double migration: muscles migrate over the epithelium and PCs migrate over the muscles to allow coiling of the testis.

A to **C**. Disposition of the tissue layers and the correspondent position of the testis and genital disc derivatives (**A**' to **C**') **A.** Histologic composition of gonads and genital disc and their location at 3h APF. A layer of muscle cells will differentiate on the genital disc (**B** and **B'**) *dsx* and JNK signalling are required in the PCs and Notch signalling, *Abd-B*, *Six4* and *Dr* in the muscles for correct development. **C.** Muscle fibres from the genital disc migrate towards the gonad and PCs slide from the gonad towards the end of vas deferens after the contact of gonads and derivatives of the genital disc. The role of ECM components is key at this stage (**C'**). Testes continue to elongate and coil (**D'**) for 12h, when there is an outer layer of PCs under which a muscle layer is located (**D**). The elongation and coiling correlates with spermatogenesis and the activation of sperm is only produced after a striated muscle contraction.



D. 3.1 Pigment cells provide a microenvironment for muscles determination.

Pigment cells actively transcribe *SOX100B*, an orthologous of *SOX9* in mammals Sertoli cells and whose role is not only informing the stem cell population to differentiate or to remain as stem, but also to alert if there is an inconsistency in sex determination, for example in Turner syndrome (Ramathal et al., 2014). Taking into account the similarities observed in the genetics of Pigment and Sertoli cells, and the phenotype of our combinations, when we altered the PCs, we can conclude that PCs act as Sertoli cells, allowing division and differentiation of the muscle but also, and more important, alerting if there are sex discrepancies. Even if we did not see the expected phenotype when altering the PCs specification, our results when altering JNK, sex or ECM components in the PCs demonstrate that they may be a good model for the study of Sertoli Cells.

Contrary to Falco et al., (2008), who observed that pigment cells would disappear at embryonic stages, for example, in *Wnt2*, *SOX100B* and *eye absent* (*eya)* mutants, we continue to observe pigment cells at adult stages in these mutants, even in combinations where sex was changed in the PCs (Fig. R. 20). In these cases, we observed white testes that have failed to elongate and coil, and whose sarcomere organization had parallel and perpendicular fibres, the pattern expected in a striated muscle.

Alteration in the JNK signalling-pathway also triggered changes in testis pigmentation and changes in testis morphology. Testes would be shorter and uncoiled; we also observed that the sarcomere organization would be more similar to a striated fibre than to a smooth muscle. We initially thought that the absence of coiling could be due to a JAK/STAT alteration in the hub, since JAK-STAT is active there, which may have prevented normal spermatogenesis. However, the inhibition of JAK/STAT did not show similar phenotypes, and therefore we propose that the phenotypes observed after the inhibition of JNK may be related to its role in ECM modification, to allow migration, or to the activation of EGFR pathway in the close-by tissue, the smooth muscle, triggering its contraction (Hong et al., 2014) and promoting myoblast differentiation

Regarding the pigment production, it is clear that pigmentation needs to have PCs, but PCs may not develop pigmentation in every portion of the male reproductive system. It is believed that pigmentation in part of the male reproductive system allows female-male species recognition (Hubby and Throckmorton, 1960 figures. I. 4. 1 and I. 8) but considering our fertility results, indicating that pigment absence may only explain a small percentage of the infertility in mutants (R. 3 and Fig. R. 26), we conclude that pigment is not so relevant for fertility. However, this is what happens in our in laboratory

experiments, where there is hardly any chance of inter-specific crosses, something that, by contrast, occurs in nature, where populations of different species may share the same ecosystem at the same time (Mercot et al., 1994; Atkinson and Shorrocks, 1977).

D. 3.2 Muscle type and its impact on fertility.

We have observed that the muscle surrounding the testis is mostly of smooth shape (Susic-Jung et al., 2012) but sometimes there are a few striated fibers too. This fact suggests that either the muscle is not well defined or some striated muscle fibers may migrate from a close structure, for example from the hindgut, as we have observed when we inhibited cell division at the *cad* region (Fig. R. 6). What seems clear is that changes in muscle determination (Fig. D. 1 B) leads to uncoiled testes and a change of structure from smooth towards striated, which in turn relates to infertility and, as previously discussed, perhaps to an early sperm activation that might puncture the testis.

Therefore, it is interesting to understand the autonomous determination of the muscle. We have depleted many muscle selector genes, some of them required for visceral muscle (Fig R. 9) and found wildtype testis muscle. Nonetheless, we have described that the requirement of *Six4*, *Dr* and *Abd-B*, as well as its sexual specificity, make this muscle unique. As mentioned before, *Six4* and *Dr* are known to be muscle selector genes; however, *Abd-B* was not reported before to have such a role. We have found that *Abd-B* is active in vas deferens and testis muscle and that when inhibited, testes are not coiled and sarcomere organization changes from smooth towards striated. *Abd-B* not only defines abdominal fate, but also it is active in the male germline, where it may be a target for the JAK/STAT pathway involved in the correct spermatogenesis (Papagiannouli and Lohmann, 2015). We have proposed that spermatogenesis might force elongation: a long structure requires a long container; therefore, the differentiation of spermatid into spermatozoids and the acquisition of the long tail may push the neighbouring cells posteriorly. *Abd-B* is also known to interact with integrins (Schardt et al. 2015) and alter actin based cytoskeleton in the ovary through activation of *spalt*, *cut* or *ems* (Gandille et al., 2010), and may thus contribute to testes elongation through muscle specification.

We have also found alterations in the testis muscle when changing their sex. It seems clear that this unique muscle, only found in males, may be highly responsive to changes in its sex. When we altered sex determination in muscle by activation of *tra*, which triggers expression of *DsxF* instead of *DsxM,* we observed that muscles looked striated. This effect might be due then to an autonomous effect but also be mediated by signaling cascades.

Abd-B has been shown to be upstream of *dsx* in abdomen development (Wang and Yoder, 2012; Foronda et al., 2012), so this may also occur in the testis muscle

Although Kozopas et al. (1998) showed that muscles migrate under the pigment cells and that both PCs and muscles needed *Wnt2* for their correct migration and/or differentiation, they were not able to determine if the testis malformations observed were due to the alteration of PCs or muscles, as they affected both tissues at the same time, or even if the *Wnt2* involvement in muscle differentiation might be through its role in the nervous system (Liebl et al., 2010). Our results have shown that changes in the expression of sex-related genes specifically in PCs, from male towards female, may alter muscle fate from smooth towards striated (Fig. R. 21). We propose then, that PCs provide the correct niche for muscle cell-fate decision and therefore act as a non-autonomous cue for muscle specification (Fig. D. 2 compare to Fig. I. 9. A).

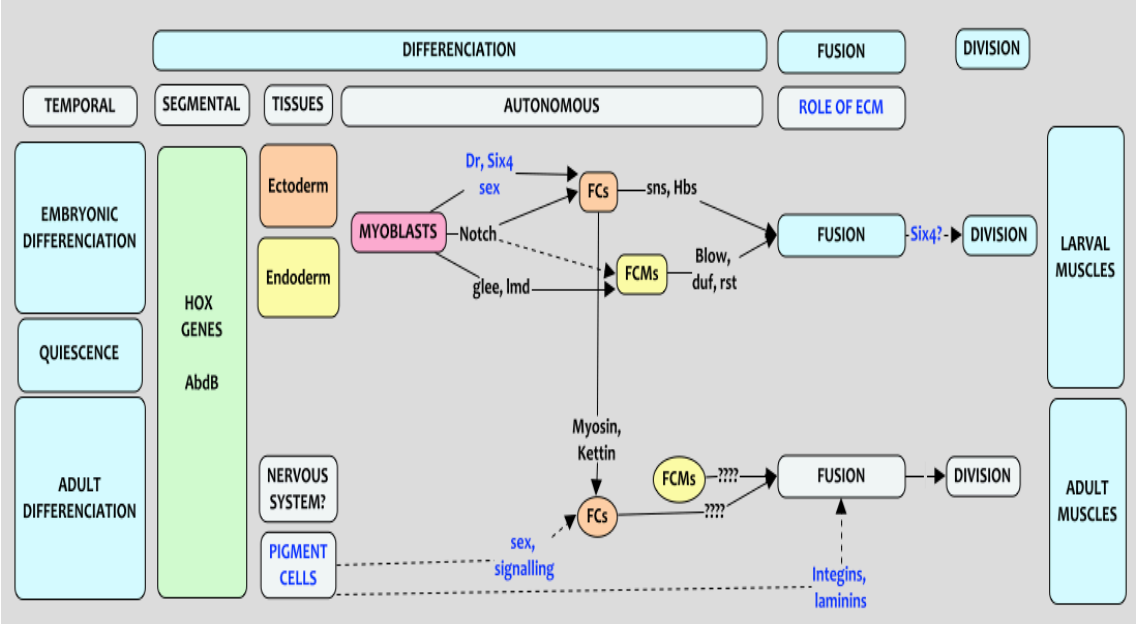


Fig. D. 2. Autonomous and environmental cues, from segments and tissues, that determine muscle cell fate and differentiation. The information derived from this thesis is depicted in blue.

We have also found Notch signalling activation in the muscle, which may indicate its role in myoblast fusion or in the activation of *Six4* (Assa-Kunik et al., 2007). Myoblasts cease to express or lower their level of Notch expression once they are in the close vicinity to fusion-competent myoblast (Engel and Rand, 2014) and lowering the levels of Notch would accelerate the fusion and differentiation of the muscles (Gildor et al., 2012). Nevertheless, the higher Notch activity we have found may be related to the finding of a possible muscle stem-like adult muscle precursors (AMPs) niche (Anant et al., 1998;

Figeac et al., 2007; Piccirillo et al., 2014) at the position where the vas deferens contacts the ejaculatory duct (Fig. R. 16). These AMPs may contribute to normal muscle development and regeneration in pathological conditions, and therefore their correct specification is, again, crucial. AMPs populations in Drosophila have been described in flight muscles (Anant et al., 1998; Gunage et al., 2014) and midgut (Jiang and Edgar, 2009), but so far none in the reproductive system.

D. 3.3 The role of ECM in the coiling of the testes.

We have seen similar phenotypes when depleting ECM components in PCs and smooth muscle (Fig. R 24 and R. 25), which highlights the ECM as important in communicating the two tissues. The elimination of integrins in the muscles triggered similar phenotypes as when the division of muscle was partially inhibited, pointing out to the role of integrins in muscle attachment. As explained before, integrins are tightly related to laminins, which in turn are required for rapid cell movements, as in migration. Considering that during development there are some temporal windows where a specific event has to take place in a specific time, it is not a wild guess to assume that an alteration in integrin or laminin disposition during metamorphosis may trigger the observed malformation phenotypes.

D. 4 Relevance of the reproductive system as a model

Up to here, we have discussed the formation of the adult male reproductive system of *Drosophila melanogaster*, how two organs contact although being initially at a long distance apart, and how the two outer tissues of the testes communicate to allow the correct elongation of the testis to ensure fertility. We have also presented the muscle of the testis as a sensitive tissue that, whether it is smooth or striated, it responds to changes in its close environment, especially to sex alterations. Has this information any practical implication?

The discovery of substances that act as hormone competitors has had an impact on the environment and human health, and led to their being banned in industry (Munn et al., 2003), This has triggered economic consequences for the plastic industry, for example, the prohibition of Bis-phenol-A (BPA) in the baby feedings bottles forced the industry to investigate new substances with similar properties. The plastic industry relies on organic molecules to improve plastic properties such as clarity and toughness to use a lesser amount of plastic. However, some of those components have been proved to be endocrine disrupting chemical (EDC) that cause feminization above all in water ecosystems where

they accumulate (Mansilha et al., 2013; Atli, 2013). It would be a great advantage to know beforehand which of those components may alter hormonal signalling. Some animals have been use as EDC detectors, such as zebrafish (Caballero-Gallardo et al., 2016). We think that Drosophila male reproductive system may be a good model to test the possible danger to sexual development in humans of some of these products. We base this idea on the rapid response of PCs and testis muscles to feminization and the easy handling and rapid development of this insect, what would allow to perform analyses involving exposure to increasing amounts of hormonal disruptors along generations.

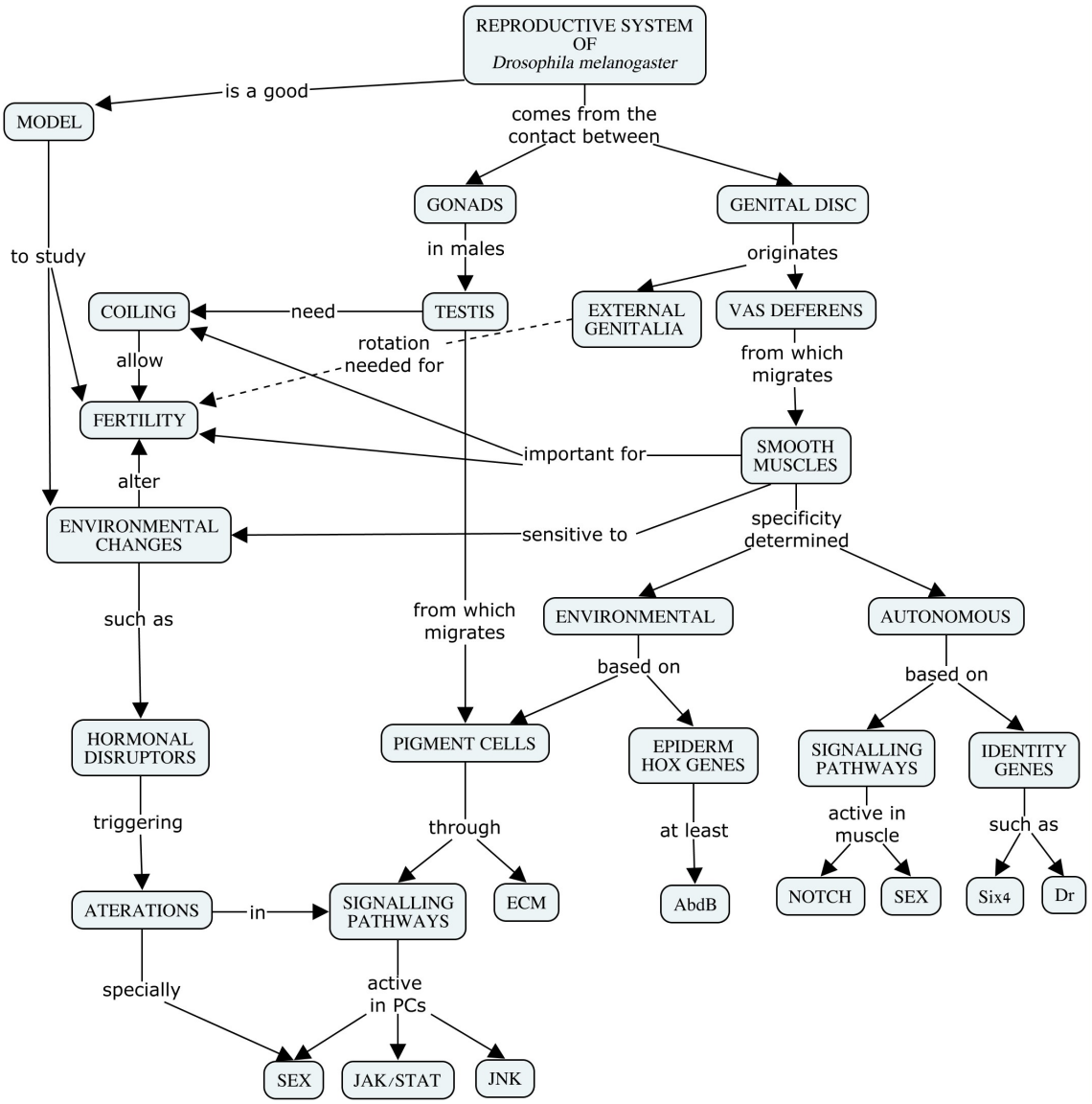


Fig. D. 3. Diagram of the main findings in this thesis and how do they relate to each other.

CONCLUSIONS

1. Gonads and genital disc contact during pupal stages to form the reproductive system. The genital disc evaginates autonomously in the absence of the gonads. However, testes need genital disc contact to coil.
2. The contraction of the smooth muscle surrounding the testis pushes the testis to elongate and coil. All the testis muscles come from the vas deferens, which evaginates from the genital disc. There is a small muscle stem-cell population at the position where the vas deferens contact with the ejaculatory duct.
3. Smooth muscle autonomous determination depends on the *Sine oculis-4*, *Drop* and *Abdominal-B* genes, but it also depends on cell-autonomous sex-specification and its microenvironment has a great impact in its final fate.
4. Pigment Cells are needed for environmental muscle determination and they are maybe also needed to maintain fertility as they protect germ cells and allow reproductive isolation, based on female recognition of pigment color.
5. Pigment cells do not divide, therefore, they stretch and their area increases to cover the full-volume adult testis and the vas deferens.
6. The extracellular matrix allows the muscle fibres attachment (by integrins) and the communication between testis smooth muscle and pigment cells (by laminins) providing the structure through which they can communicate (*trol* and *vkg*).
7. JNK is active in the Pigment Cells where it activates JAK/STAT, which triggers Diap1 activation, preventing therefore apoptosis in the Pigment Cells.
8. Notch is active in the muscles, where it might control their identity and may be required during migration.
9. Changes in the autonomous muscle specification and in its close environment lead to changes in sarcomere organization, from a smooth-like structure towards a striated like. Particularly, changes in the sexual identity trigger conspicuous effects, therefore it is a good model to test the effect of substances as Endocrine Disruptors (EC).

CONCLUSIONES

1. Las gónadas y el disco genital contactan durante los estadios pupales para formar el aparato reproductivo. El disco genital evagina de manera autónoma e independiente de la ausencia de las gónadas, tanto en hembras como en machos. Sin embargo, los testículos necesitan del contacto del disco genital para espiralizar.
2. La contracción del músculo liso que rodea el testículo empuja al testículo en su elongación y espiralización. Los músculos del testículo provienen del vas deferens que evagina desde el disco genital. Hay una pequeña población de células madre musculares en la base del vas deferens, en la zona de contacto con el ducto eyaculatorio.
3. La determinación autónoma del músculo liso depende de los genes *Sine Oculis-4*, *Drop* y *Abdominal-B*, pero su microentorno más cercano tiene un gran impacto en su destino celular final.
4. Las Células Pigmentarias son necesarias para la determinación no autónoma de los músculos del testículo y se requieren para mantener la fertilidad, ya que además de proteger las células germinales y podrían permitir el aislamiento reproductivo entre especies, basado en la detección de los diferentes colores que puede adoptar el pigmento en las distintas especies.
5. Las Células Pigmentarias no se dividen, por lo que su superficie aumenta para cubrir el testículo adulto y el vas deferens.
6. La matriz extracelular podría proporcionar el soporte para la migración (*vkg* y *trol*), colabora en la adhesión de las fibras musculares (integrinas) y comunicación entre el músculo liso del testículo y las Células Pigmentarias (lamininas).
7. La vía de señalización JNK está activa en las Células Pigmentarias donde activa JAK/STAT, que a su vez activa la transcripción de *Diap1*, evitando así, la apoptosis de las Células Pigmentarias.
8. Notch está activo en los músculos del testículo, donde controla su diferenciación y puede ser necesario durante la migración.
9. Cambios en la determinación autónoma del músculo y de su entorno cercano provocan cambios en la disposición de las fibras de la sarcómera, pasando de una estructura asimilable a músculo liso a una estructura similar a la del músculo

estriado. En particular, los cambios en la identidad sexual tienen efectos muy llamativos, lo que lo convierte en un modelo excelente para comprobar el efecto de sustancias como disruptores hormonales.

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AKNOWLEDGEMENTS